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The Effects of Chorioallantoic Grafts on the Developing Chick Embryo

I. Studies on Weight and Histology of Homologous and Heterologous Tissues

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WITH ONE PLATE

INTRODUCTION

THE grafting of tissues to the chorioallantoic membrane (CAM) of the chick embryo has been widely used for study of organ-specific growth stimulation. Murphy (1916) and Danchakoff (1916) first observed that chorioallantoic grafts of adult chicken spleen induced enlargement of the spleens of host embryos. The former attributed spleen hypertrophy to an increase in the number of small lymphocytes while the latter attributed it to an intense proliferation of lymphoid haemocytoblasts which ultimately differentiated into granulocytes. In a subsequent study Danchakoff (1918) observed that transformation of mesenchyme into granuloblastic cells was not confined to the spleen but extended throughout the whole mesenchyme of the host.

An extensive investigation of the problem of the effect of CAM grafts of adult chicken tissue on homologous tissues of the host embryo was carried out by Ebert (1955). He observed a very marked enlargement of spleens in host chicks following grafts of adult chicken spleen (Ebert, 1951). In other experiments using CAM grafts labelled with radioactive methionine, Ebert (1954) observed that enlarged spleens had an increased nitrogen content and a higher specific radioactivity than the kidney and liver. The DNA content of enlarged spleens did not differ significantly from control spleens. On the basis of these observations he suggested that enlargement of the spleen was due to increased protein content; that transfer from the graft to the host was tissue-specific and there was a selective incorporation of tissue-specific proteins from grafts to homologous tissues rather than transfer of whole cells. A transfer of such specific protein moieties was shown by Walter *et al.* (1956). Following injection of either a clear super-

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natant of S^{35}-labelled homogenized liver or heart into 9-day-old embryos, a higher specific activity was observed in the respective host tissues of liver and heart. Similar studies have also been conducted by Ebert (1957; 1958 *a*, *b*). This investigator was able to demonstrate the localization of tissue-specific fractions in homologous organs following intravenous injections of cytoplasmic materials. Selective localization was obtained following the injection of microsomal and supernatant fractions, but not with nuclear and mitochondrial fractions. On the other hand, Van Haeften (1958) observed significant enlargement of the host spleen following grafting of cell-free homogenates of spleen to the CAM.

An alternative hypothesis, that of a 'graft-*versus*-host reaction', has been advanced to explain splenic enlargement following introduction of adult spleen or blood-cells into the chick embryo. Simonsen (1957) observed that 18-day chick embryos, injected intravenously with adult chicken spleen-cells or blood-cells, manifested symptoms of severe haemolytic anaemia, and that marked hypertrophy occurred in the spleen, liver, and thymus about 2 weeks after hatching. He further observed that reticulo-endothelial cells replaced erythropoietic and myelopoietic cells in the bone-marrow. The enlargement of the spleen, the haemolytic anaemia, and the histological changes in the bone-marrow he attributed to an immune reaction of the injected cells against the host and colonization of the host's tissues by injected cells. Recently, Cock & Simonsen (1958) have shown that when blood from one inbred line of adult chickens was injected into newly hatched chicks of a cross between two highly inbred lines, gross enlargement of the spleen and liver occurred. On the other hand, when F_1 blood was injected into F_1 chicks only a relatively slight enlargement occurred, and this they attributed to the antigenic diversity within one of the inbred lines. Terasaki (1959) has shown that when adult chicken lymphocytes were injected into chick embryos there was a marked splenic enlargement in the host. On the other hand, adult monocytes and thymocytes did not cause significant enlargement of the spleen. Billingham & Brent (1957) also attribute the production of 'runt disease' in mice and the mortality observed following injections of A-strain mice with C57 spleen-cells to the immunological reactions produced by the inoculated adult spleen-cells against the tissue antigens present in their young hosts.

Evidence of a cellular immune reaction on the part of adult spleen-cells in an embryonic environment has been demonstrated in the chick by Ebert (1957; 1958 *a*, *b*) and in the larval salamander by De Lanney (1958). The former investigator observed that following intracoelomic grafts of adult spleen on the 4th day of incubation, a profound effect was produced on the vascular system, leading to death in 30 per cent. of the host embryos. The latter investigator observed that when adult salamander spleen was grafted into a pocket in the dorsal fin or in the coelom of the larval salamander, *Taricha torosa*, growth of the host spleen was suppressed.

From this review of the literature it would seem that in order to ascertain if organ-specific growth stimulation exists some organ should be studied which is not known to be engaged in haematopoiesis. The histogenesis of the duodenum and histochemistry of the mucopolysaccharides in the connective tissues of this organ have been described by Van Alten & Fennell (1957). The objectives of the present investigation were to study: (1) the effects of chorioallantoic grafts of embryonic duodenum and various adult organs (spleen, liver, heart, skin, brain, and duodenum) upon the weight of homologous and heterologous organs in the developing chick embryo; (2) the effects of soluble extracts of adult and embryonic chicken organs on the host embryo when injected into the yolk sac; and (3) the histology of the various embryonic organs following CAM transplants.

MATERIALS AND METHODS

Chickens of both sexes, of different breeds and of ages ranging from 3 months to about one year after hatching were used as donors of tissues for grafting. Embryonic duodena were obtained from embryos incubated 15, 16, 17, 18, and 20 days.

The procedure of the chorioallantoic transplantations is given in detail by Van Alten (1958). The approach has been to compare weight differences of the total host embryo and various host organs (spleen, liver, heart, intestine, and duodenum) following: (1) chorioallantoic transplants of fresh adult organs; (2) grafts of adult chicken duodenum treated with 95 per cent. ethanol at —20° C. for 24 hours, lyophilization, or heat (80° C. for 20 minutes); (3) sham operations in which the complete chorioallantoic transplantation procedure was carried out, but only a drop of sterile Ringer's solution, or a small piece of 2 per cent. agar, was put on the CAM; (4) transplants of adult rat duodenum; and (5) grafts of 15-, 16-, 17-, 18-, and 20-day embryonic duodena.

The chicken was killed by decapitation and various tissues (duodenum, spleen, liver, heart, skin, and brain) were quickly removed and cut into small (2–3 $mm.^3$) pieces in chick Ringer's solution. In order to sterilize the duodenum it was placed in 200 ml. of chick Ringer's solution containing 1,000 mg. of chloromycetin for 10 minutes. In order to determine if the tissue was sterilized an occasional piece of tissue was streaked on a blood-agar plate. In no case was contamination found. One of the prepared pieces of tissue was then placed at the bifurcation point of blood-vessels on the CAM. The above procedure was also used for adult rat duodenum.

In order to ascertain the effect of soluble cell-free material of the duodenum, liver, and heart of the adult chicken on the chick embryo, the following procedure was carried out. Tissues, either adult or 20-day embryonic chick, were homogenized in a Waring blendor with a 1 to 5 ratio of 0·15 M saline (buffered to pH 7·4 with 0·005 M phosphate buffer) for 20 minutes at 4° C., after which the homogenate was centrifuged at about 500 g. for 30 minutes at 4° C. The supernatant after Seitz filtration was used for injection into the yolk sac.

The statistics used in comparing the weight of the chick, spleen, liver, heart, intestine, and duodenum following various treatments to the CAM, were an analysis of variance (Dixon & Massey, 1951) and the multiple range test for heteroscedastic means (Duncan, 1957).

Following various CAM grafts, host tissues of spleen, liver, heart, duodenum, and CAM were fixed in Bouin's fixative for 24 hours and embedded in paraffin by routine methods of tissue preparation. Tissue sections were cut at 5 μ. All tissues were routinely stained with haematoxylin and eosin, Gomori's trichrome stain (Gomori, 1950), the triple stain of Himes & Moriber (1956), and the Azure II eosin procedure (Lillie, 1954). For elastic tissue Weigert's resorcin fuchsin was used and Van Giesen's picro-acid fuchsin was used for collagenous tissue.

RESULTS

The effect of chorioallantoic transplants of adult and embryonic chicken tissues on the weight of homologous and heterologous tissues of the host embryo

It is evident from Table 1 that, following adult duodenal grafts, there was a significant reduction in weight of the whole embryo and a marked enlargement of the spleen, liver, and heart, and that there was no significant weight difference in the intestine or duodenum. It can also be seen that after liver grafts the liver and heart showed a significant enlargement over those of controls (sham operation). However, the liver and heart were significantly larger after grafts of duodenum than after liver grafts. When alcohol-inactivated adult chicken duodenum was transplanted to the CAM it was observed that there was a decrease in weight of both the whole embryo and the duodenum.

TABLE 1

Fresh weights of whole embryos and of homologous and heterologous organs following chorioallantoic transplants

	Mean weight of host and host organs						
Treatment	*No.*	*Whole chick*	*Spleen*	*Liver*	*Heart*	*Intestine*	*Duodenum*
		(g.)	(mg.)	(g.)	(g.)	(g.)	(mg.)
A. Grafts of adult chicken duodenum .	33	16·25	36·41	0·6609	0·2115	0·3963	67·08
B. Grafts of adult chicken liver . .	29	18·13	16·97	0·5421	0·1786	0·4148	67·08
C. Sham operation .	25	18·86	12·00	0·4716	0·1592	0·3924	61·96
D. Grafts of inactivated adult chicken duodenum . . .	21	17·60	13·19	0·4514	0·1448	0·3757	55·37
Statistical F test . .		9·41*	22·19*	30·77*	28·75*	0·75	5·72*
Statistical multiple range test[1]. . . .		(BC) (BD)	(BCD)	(CD)	(CD)	(ABCD)	(ABC) (CD)

[1] Any two means appearing together within the same parentheses are *not* significantly different at the 5 per cent. level.

In Table 2 it is evident that the spleen and heart were significantly larger when brain was grafted to the CAM than when control procedures were used. The duodenum was larger, but other significant growth changes were not observed after grafts of rat duodenum.

TABLE 2

Fresh weights of whole embryos and of homologous and heterologous organs following chorioallantoic transplants

		Mean weight of host and host organs					
Treatment	*No.*	*Whole chick*	*Spleen*	*Liver*	*Heart*	*Intestine*	*Duodenum*
		(g.)	(mg.)	(g.)	(g.)	(g.)	(mg.)
A. Grafts of adult chicken duodenum . .	19	20·63	54·66	0·7158	0·2121	0·5778	58·73
B. Grafts of adult chicken brain .	28	23·26	17·49	0·5681	0·2026	0·6143	58·60
C. Grafts of adult rat duodenum .	21	22·58	9·89	0·5267	0·1590	0·5700	62·93
D. Sham operation	26	22·53	9·83	0·5142	0·1546	0·5692	55·77
Statistical F test .		7·05*	75·91*	24·29*	5·67*	1·43	3·12*
Statistical multiple range test[1] . .		(BCD)	(CD)	(BC) (CD)	(AB) (CD)	(ABCD)	(ABC) (ABD)

[1] Any two means appearing together within the same parentheses are *not* significantly different at the 5 per cent. level.

TABLE 3

Fresh weights of whole embryos and of homologous and heterologous organs following chorioallantoic transplants

		Mean weight of host and host organs					
Treatment	*No.*	*Whole chick*	*Spleen*	*Liver*	*Heart*	*Intestine*	*Duodenum*
		(g.)	(mg.)	(g.)	(g.)	(g.)	(mg.)
A. Grafts of adult chicken duodenum . .	20	21·36	43·31	0·7280	0·2405	0·6285	64·72
B. Grafts of adult chicken lyophilized duodenum.	38	23·16	13·89	0·5253	0·1861	0·5737	54·19
C. Sham operation	28	23·35	13·10	0·5393	0·1636	0·5925	64·06
Statistical F test .		4·86*	103·21*	36·88*	14·29*	3·33	10·52*
Statistical multiple range test[1] . .		(BC)	(BC)	(BC)		(ABC)	(AC)

[1] Any two means appearing together within the same parentheses are *not* significantly different at the 5 per cent. level.

It is evident on the basis of the multiple range test (Table 3) that, following adult duodenal grafts, the weight of the whole embryo was significantly less and the weights of the spleen, liver, and heart were significantly more than after lyophilized duodenal grafts, as well as (in agreement with Table 1) more than in controls. On the other hand, when lyophilized duodenum was placed on the CAM the weight of the host heart was significantly higher, while the duodenum was significantly lower than in controls.

Table 4 again confirms the effects of a graft of adult chicken duodenum. Following grafts of either heated or alcohol-extracted chicken duodenum there were no significant weight differences from controls in either the whole embryo or the individual organs.

TABLE 4

Fresh weights of whole embryos and of homologous and heterologous organs following chorioallantoic transplants

		Mean weight of host and host organs					
Treatment	*No.*	*Whole chick*	*Spleen*	*Liver*	*Heart*	*Intestine*	*Duodenum*
		(g.)	(mg.)	(g.)	(g.)	(g.)	(mg.)
A. Grafts of adult chicken duodenum . . .	34	23·45	46·63	0·7229	0·2185	0·6412	66·51
B. Grafts of heated adult chicken duodenum (80° C. for 20 minutes)	30	24·85	13·22	0·5713	0·1917	0·6607	73·43
C. Grafts of alcohol extract of adult chicken duodenum . . .	36	24·41	12·38	0·5494	0·1853	0·6142	66·64
D. Sham operation . .	27	24·81	12·05	0·5378	0·1800	0·6622	71·77
Statistical F test . .		4·09*	36·46*	25·77*	8·75*	2·13	3·95*
Statistical multiple range test[1]		(BCD)	(BCD)	(BCD)	(BCD)	(ABCD)	(AC) (BD)

[1] Any two means appearing together within the same parentheses are *not* significantly different at the 5 per cent. level.

TABLE 5

Fresh weights of whole embryos and of homologous and heterologous organs following chorioallantoic transplants

		Mean weight of host and host organs					
Treatment	*No.*	*Whole chick*	*Spleen*	*Liver*	*Heart*	*Intestine*	*Duodenum*
		(g.)	(mg.)	(g.)	(g.)	(g.)	(mg.)
A. Grafts of adult chicken heart. . . .	19	24·89	16·06	0·5684	0·1763	0·6221	71·11
B. Grafts of adult chicken skin	25	24·97	28·86	0·6540	0·1868	0·6688	82·32
C. Sham operation . .	35	25·32	14·07	0·5640	0·1871	0·6737	72·11
Statistical F test . .		0·37	7·54*	6·25*	1·85	2·14	3·38*
Statistical multiple range test[1]		(ABC)	(AC)	(AC)	(ABC)	(ABC)	(AC)

[1] Any two means appearing together within the same parentheses are *not* significantly different at the 5 per cent. level.

Table 5 shows that there was no significant difference from controls in the weights of whole chicks or of individual organs following heart grafts. Grafting of skin significantly increases weight of spleen, liver, and duodenum as compared with controls.

The question naturally arises as to whether enlargement of host embryonic organs following grafts of adult tissue was dependent on an increase in protoplasm or was merely a manifestation of oedema. The analysis in Table 6 indicates that the enlargement was due to an increase in dry weight, both fresh and dry weights of the spleen and heart being significantly larger following spleen and duodenal grafts. Moreover, following duodenal grafts the fresh and dry weights of the liver were significantly increased. The dry weight of the duodenum was not significantly different from the controls after either duodenal or spleen grafts. Duodenal grafts produced a greater weight increase of host organs than did spleen grafts.

TABLE 6

Fresh and dry weights of homologous and heterologous organs following chorioallantoic transplants

	Mean weight of host organs									
Treatment	*No.*	*Spleen fresh*	*Spleen dry*	*Liver fresh*	*Liver dry*	*Heart fresh*	*Heart dry*	*Duodenum fresh*	*Duodenum dry*
		(mg.)	(mg.)	(g.)	(g.)	(g.)	(g.)	(mg.)	(mg.)
A. Grafts of adult chicken duodenum . .	25	32·65	9·66	0·7014	0·1598	0·1733	24·84	91·83	12·58
B. Grafts of adult chicken spleen	25	24·04	5·06	0·5930	0·1383	0·1515	21·75	90·62	11·56
C. Sham operation	28	13·37	2·33	0·5909	0·1424	0·1250	17·97	103·66	12·49
Statistical F test .		21·69*	4·44*	139·57*	5·29*	45·79*	25·64*	3·71*	2·77
Statistical multiple range test[1] .				(BC)	(BC)			(AB)	(ABC)

[1] Any two means appearing together within the same parentheses are *not* significantly different at the 5 per cent. level.

Tables 1–3 show that the weight of the whole chick following duodenal grafts was lower than that of control embryos, and that the weight of the duodenum after duodenal grafts was not significantly different from that of controls. The question arises as to whether the relative weights of duodena following duodenal grafts are significantly higher than those of the controls. This was tested by covariance analysis of the data in Table 1. An F_1 value, which measures the difference between the two sample regression coefficients, was found to be 6·6 with df. = 1, 51, which, at the 95 per cent. level, is significant. This clearly indicates that the weight of the duodenum following duodenal grafts is significantly larger than in the controls when allowance is made for the difference in body-weight.

Since transplantation of adult tissue to the CAM induces profound changes in various organs of the embryo, the question arises as to whether or not grafts of embryonic tissue would have the same effect. Since the adult duodenum was the most effective stimulant for host tissues, duodena from various ages of embryos were transplanted to the CAM.

The multiple range test in Table 7 shows that the weight of the whole chick following grafts of 16-day embryonic duodenum is significantly higher than the weight of embryos following grafts of 18- and 20-day duodena but is not significantly different from control experiments. However, the weight of the whole chick following 18-day embryonic duodenal grafts is significantly less than that of the controls. Further, compared with the controls, the weights of the spleen, liver, heart, and intestine are not significantly higher following grafts of 16-, 18-, or 20-day embryonic duodena. However, after grafting 20-day chick embryonic duodenum the weight of the host duodenum is significantly decreased, but it is not significantly different from controls after grafting 18- and 16-day duodena.

TABLE 7

Fresh weights of whole embryos and of homologous and heterologous organs following chorioallantoic transplants

Treatment	*Mean weight of host and host organs*						
	No.	*Whole chick*	*Spleen*	*Liver*	*Heart*	*Intestine*	*Duodenum*
		(g.)	(mg.)	(g.)	(g.)	(g.)	(mg.)
A. Grafts of 16-day chick embryo duodenum .	24	26·13	13·47	0·5908	0·1817	0·6613	74·65
B. Grafts of 18-day chick embryo duodenum .	24	24·66	13·05	0·5717	0·1796	0·6658	67·70
C. Grafts of 20-day chick embryo duodenum .	37	24·86	12·72	0·5630	0·1768	0·6286	60·88
D. Sham operation	22	25·56	11·62	0·5577	0·1709	0·6541	71·39
Statistical F test .		5·07*	1·62	2·33	0	0·875	8·75*
Statistical multiple range test[1] . .		(AD) (CD) (BC)	(ABCD)	(ABCD)	(ABCD)	(ABCD)	(AD) (BD)

[1] Any two means appearing together within the same parentheses are *not* significantly different at the 5 per cent. level.

The effect of extracts of adult and embryonic chicken organs on the host embryo when injected into the yolk sac

The supernatant of homogenates of 0·1 ml. of adult chicken duodenum, liver, and heart were injected into the yolk sac of 96-hour embryos; controls were injected with 0·1 ml. of 0·15 M saline. A mortality rate of 100 per cent. followed the injection of supernatant of homogenized duodena. The time of death varied with the amount of the material injected. The results of this experiment are summarized in Table 8.

TABLE 8

The effect of soluble antigens on the host embryo when injected into the yolk sac

Amount (mg. wet wt.)	*Time of death*
(mg.)	(hours)
20	24
10	36–48
7·7	48–60
6·7	56–72

Similar results, i.e. the death of all embryos, were observed following the injection of adult chicken liver and heart and 20-day embryonic duodenal supernatant. This mortality could not be attributed to pressure because the control embryos survived until the 20th day of incubation, at which time they were harvested. The supernatant and yolk of eggs which had just died were streaked on blood-agar plates. No bacterial colonies were found on these plates so death could not be attributed to infection.

Morphology of chick embryos and various homologous and heterologous organs after chorioallantoic transplantation of adult and embryonic chicken tissues

Whole embryo. It was observed that embryos harvested on the 18th day of incubation after grafting of adult chicken duodenum were small and appeared pale. When adult chicken spleen had been transplanted a few chicks appeared pale, but none as pale as after duodenal transplants. All embryos appeared normal in the controls and after grafts of adult chicken liver, heart, brain, skin, the variously treated duodena, and embryonic duodenum.

Duodenum. The gross morphology of the duodenum appeared essentially the same following transplants of adult and embryonic chick tissue and after control procedures, but the microscopic structure of the duodenum was somewhat altered following grafting of adult duodenal tissues (Plate, fig. A). Following grafting of adult duodenum, the connective tissue within the villi consisted of a compact mesenchymal layer until the 15th day of incubation, and this was succeeded by the lamina propria mucosae on the 16th day. Van Alten & Fennell (1957) identified the lamina propria mucosae on the 17th day under normal conditions. Further, Van Alten (1955) found goblet cells first on the 17th day of incubation in untreated embryos; however, following adult duodenal grafts, goblet cells were identified in great abundance on the 16th day. These observations indicate that duodenal grafts accelerated differentiation of the duodenal tissues of the host embryo.

After grafting either adult chicken duodenum or spleen there was an infiltration of lymphocytes into the duodenal tissue, but this was not observed following liver, heart, brain, skin, or embryonic duodenal grafts (Plate, fig. B).

It is evident from the results summarized in Table 9 that transplantation of adult duodenum to the CAM accelerates the histochemical differentiation of both connective tissues and goblet cells in the embryonic duodenum.

Table 9

The differentiation of polysaccharides in duodenal tissues following CAM adult duodenal grafts

Treatment	*Muscle connective tissue*	*Lamina propria mucosae*	*Submucosa*	*Glands and goblet cells*
	Age of embryo in days			
Control	15	18	18	17
Adult chicken duodenum	13	17	17	16

All tissues were stained by the Hotchkiss–McManus PAS reaction.

Spleen. Spleens of host organs following duodenal grafts were markedly enlarged with a diameter of about 10 mm., the maximum in control embryos being 2 mm. The surface of enlarged spleens exhibited numerous white tumour-like protuberances. Essentially the same type of reaction occurred following spleen and skin grafts. On the other hand, spleens of control chicks, and host spleens after grafts of heart, liver, inactivated duodena, and embryonic duodena (15-, 16-, 17-, 18-, and 20-day embryos), were spherical and dark reddish brown in colour.

In sections of enlarged spleens of 18-day embryos which had received CAM grafts of adult chicken duodenum, spleen or skin, marked changes were observed (Plate, fig. D). There were fewer venous sinuses than in the control spleens (Plate, fig. C). Within the pulpa there were few reticular cells, i.e. mesenchymal cells, and many haemocytoblasts were observed. Large nodular areas (as many as six in a single section) filled with granulocytes were walled off by reticulo-endothelial cells, and multinucleated giant cells were found to be quite numerous and uniformly distributed throughout these enlarged spleens. In contrast to control spleens, which contained many erythrocytes within the venous sinuses, very few erythrocytes were observed in enlarged spleens.

Liver. On the 18th day of incubation the liver exhibited what Kingsbury *et al.* (1956) described as a deep sulphur-yellow colour. This was essentially the appearance of the liver following CAM transplants of adult chicken spleen, liver, heart, brain, inactivated (by heat, lyophilization, or alcohol) duodenum, and embryonic duodenum. Following grafts of adult skin, the liver of host embryos (18th day of incubation) was buff coloured, with small red spots of about 2 mm. in diameter uniformly distributed over the entire surface. Following

CAM grafts of adult chicken duodenum the host livers were a bile-green colour and friable, and usually exhibited several relatively large grey areas.

By the 18th day of incubation, in both control and treated embryos, lymphocytes existed as small nodules in the connective tissues. Microscopic examination of the enlarged green livers revealed that from a histological point of view they were essentially similar to those of control embryos. This appears rather striking in the light of the gross changes which were observed.

Heart. The heart of 18-day embryos following CAM grafts of adult duodenum was pale pinkish-grey in colour and had a length in excess of 10 mm. Hearts of control embryos of the same age had a maximum length of approximately 10 mm. and a bright pink colour. Following grafts of various tissues (adult duodenum, spleen, liver, heart, skin, brain, and embryonic duodenum) the microscopic structure of the hearts of host embryos appeared to be essentially similar to that of control embryos.

Chorioallantoic membrane. The grafting of small pieces of either adult duodenum or spleen to the CAM induced the development of numerous small tumour-like masses which were sufficiently extensive to cover most of the CAM. Following the grafting of heart tissues to the CAM haemorrhagic regions were frequently observed adjacent to or near the grafted tissue on the 18th day of incubation.

A microscopic examination of the CAM adjacent to the site of grafts of adult duodenum, spleen, liver, heart, and skin, exhibited an abundance of granuloblasts and granulocytes. Many basophilic cells were also observed there and these cells were identified as haemocytoblasts. In almost every instance there were foci of grafted tissue which had not survived and these appeared as an acidophilic mass with numerous basophilic granules (Plate, fig. F).

When embryonic duodenum from 15-, 16-, 17-, 18-, and 20-day chicks was transplanted to the CAM the integrity of the graft tissue was not destroyed; the mucosa, lamina propria, submucosa, and tunica muscularis were identifiable. The epithelium of the mucosa overlying the villi consisted of simple columnar epithelium with numerous functional goblet cells (Plate, fig. E).

DISCUSSION

The data presented above demonstrate that profound changes occurred in the chick embryo after grafting adult chicken tissues to the CAM. The host spleen was enlarged following grafts of adult spleen, duodenum, brain, and skin. The host liver was enlarged following transplants of adult liver, spleen, and duodenum, while the heart was enlarged following spleen, liver, and duodenal grafts. In all cases the adult duodenum produced the most pronounced enlargement of the embryonic organs studied. Enlargement of the host duodenum following grafts of adult duodenum could be demonstrated only on a relative basis; the weight of the whole embryo was less than that of the controls, while the weight of the duodenum was about the same.

Histological studies of the enlarged spleen showed that the enlargement was primarily caused by granulopoiesis regardless of which adult organ (spleen, duodenum, skin, or brain) was used to stimulate it. Although the gross appearance of the host liver (it was bile-green in colour) and of the heart (it was enlarged and pale) was altered following CAM grafts of adult chicken duodenum, the microscopic anatomy was similar to that of the controls.

The hypothesis of organ-specific growth stimulation as proposed by Weiss (1947) and Ebert (1955) has been based largely on the observations of Murphy (1916), Danchakoff (1916), Willier (1924), Weiss (1947), and Ebert (1951). These workers found that when a small fragment of an adult chicken organ was transplanted to the vascular bed of an embryo (either CAM or vascular area of the blastoderm) it greatly stimulated growth of the homologous embryonic organs. Weiss & Andres (1952) observed an increase in the mitotic rate of the embryonic kidney after injections of kidney brei into the CAM blood-vessels, and also in the mitotic rate of the kidney and liver following injections of mesonephric brei (Andres, 1955).

Observations made during the course of this study do not entirely support the hypothesis of organ-specific growth stimulation. Van Alten (1959) also observed that the antigens prepared from spleens stimulated by adult duodenal grafts exhibited a different antigenic pattern than control spleens, and that antigens prepared from duodena stimulated by adult spleen grafts exhibited a different antigenic pattern from control duodena. Danchakoff (1918) observed that enlargement after CAM transplants of adult spleen was not confined to the spleen but extended throughout the whole mesenchyme of the host. Andres's (1955) studies were not organ-specific after injection of mesonephric brei. He observed that mitotic indices of the liver increased 23 per cent. while the mesonephros increased 46 per cent. However, Levy (1956) was unable to demonstrate the retrogression of the mesonephros after CAM grafts of 18-day embryonic mesonephros or metanephros which would be expected if organ-specific growth stimulation had occurred. Also, Wilson & Leduc (1947) have demonstrated that a number of agents (pulped liver of mice and guinea-pigs, boiled and autolysed liver, pulped kidney, and boiled egg-yolk) produced an increase in the mitotic rates in mouse livers on the 5th day after injection. Further, Saetren (1956) has observed that after injections of macerated homologous tissue into the peritoneal cavity of rats following partial nephrectomy or removal of a portion of the liver, there was a marked inhibition of mitoses in regenerating portions of kidney and liver. Steuart (see Ebert, 1958*b*) has confirmed this observation on the kidney and, further, has shown that liver homogenate suppresses the mitotic activity in the remaining kidney stump but that kidney homogenate is more specific. However, it should be kept in mind that the systems studied by Andres and those of Wilson & Leduc, Saetren, and Steuart are quite different.

The tracer studies of Ebert (1954) showed that when labelled tissues were placed on the CAM, homologous organs had a higher specific activity than heterologous organs. However, Horn & House (1955) observed that when they injected tagged homogenates of liver, kidney, spleen, and thymus into young mice the uptake value of the spleen was consistently higher than other organs. They suggested that the spleen was the most effective organ of the reticulo-endothelial system for removing foreign protein from the circulation.

An alternative hypothesis of graft-*versus*-host reaction was proposed by Simonsen (1957) to account for the enlargement of embryonic spleens following grafts of adult spleen. Billingham *et al.* (1956) observed a 95 per cent. mortality of chick embryos following injection of adult blood. Death occurred toward the end of incubation and was attributed to an infective agent. However, Simonsen (1957) and Cock & Simonsen (1958) believe that adult spleen or blood-cells can colonize host lymphoid organs, in which they multiply and react immunologically against the host. Recently, Terasaki (1959) has shown that when adult chicken lymphocytes were injected into chick embryos there was a marked splenic enlargement in the host. On the other hand, adult monocytes and thymocytes did not cause significant enlargement of the spleen. The generalized effects, i.e. enlargement of spleen, liver, and heart, observed in these studies following grafting of adult chicken duodenum may be accounted for on the basis that this organ contains much lymphocytic tissue and thus produces a graft-*versus*-host reaction. However, this hypothesis fails to give a satisfactory explanation as to why brain grafts, which contain very few lymphocytes, produced splenic enlargement while adult liver transplants, which contain many lymphocytes, did not elicit splenic enlargement. Further, the present study also shows that adult duodenal grafts, which contain fewer lymphocytes than spleen, elicited a greater enlargement of the host spleen than did splenic grafts. However, these results are in contrast to Ebert's (1959), who obtained no splenic enlargement in the embryo after grafting of adult brain tissue and observed that adult liver grafts produced a marked stimulation (about 40 per cent. as much as adult spleen) of the embryonic spleen. The graft-*versus*-host hypothesis also fails to explain why the microscopic morphology of the adult grafted tissue was destroyed and replaced by a myeloid metaplastic centre, a type of reaction to the CAM which has also been observed by Van Haeften (1958) with cell-free material. Simonsen (1957), on the basis of direct injections of adult cells, postulates that enlargement of host spleens was due to colonization of the host organ by injected cells. Ebert (1954), on the basis of DNA content of the enlarged spleens, ruled out the transfer of whole cells. Recently, however, Ebert (1958 *a*, *b*) has re-evaluated this question in the light of experiments involving serial transplantation of stimulated spleens; he observed no dilution of growth-promoting activity by serial passage. On the basis of this observation he proffers as the simplest explanation the colonization of the host spleen by whole cells from the donor, these cells being capable of reproduction. Nevertheless, he was not able to identify large populations of adult cells in the host spleen by histological analysis and thus is unable to rule out the possibility of subcellular material as the causative agent.

Recently, Van Haeften (1958) grafted a cell-free homogenate of adult spleen to the CAM and observed hypertrophy of the host homologous organ. However, Ebert (1958*a*) did not observe stimulation of the homologous organ with cell-free material but only ‘predominant localization’. By placing spleen grafts in membrane filters, which prevent the passage of cells, Ebert (1958*b*) observed a modest stimulation of the host spleen.

It was shown in the preceding paragraphs that both the organ-specific growth stimulation and the graft-*versus*-host hypothesis fail to adequately explain the results observed following grafts of adult duodenal tissue. It may be postulated that the duodenum contains substances which are non-specific and cause various organs to enlarge. Levi-Montalcini (1952) has observed enlargement of the spleen and liver of the chick after transplantation of mouse sarcoma 37 or 180 to the allantoic vesicle. The enlarged liver was described as ‘deeply suffused with bile’. Van Alten (1959) observed an increase in the number of antigens in both the duodenum and spleen after grafts of adult duodenum. This would further indicate that some sort of non-specific effect was being produced.

Heart enlargement was probably due to a compensatory reaction related to the anaemic condition of the chicks. This anaemic condition probably was due to the fact that the spleen was almost totally given over to the production of granulocytes rather than erythrocytes. The grafts were made on the 9th day of incubation, at which time Fennell (1947) observed that the definitive erythrocytes had replaced the primitive erythrocytes to become the most numerous type in peripheral blood. Thus, spleen and liver enlargement may be due in part to removal of primitive generation of blood-cells and foreign substances from the blood vascular system. On the other hand, heart enlargement may be due, in part, to compensation.

It was also observed in the course of this study that when either normal duodenum, liver, or heart were injected into the yolk sac on the 4th day of incubation, they caused all the embryos to die within 72 hours. This observation is in keeping with that of Fennell (1947) who, using a much more dilute inoculum of minced normal liver, observed that 67 per cent. of the embryos died within 5 days after inoculation. He further observed that injections of normal liver-mince produced blood changes which meet the requirements for haemocytoblastosis.

SUMMARY

1. It was observed that following CAM grafts of adult chicken duodenum there was a marked decrease in the absolute weight of the host, a marked increase in the weight of the spleen, liver, and heart, and a relative weight increase in the duodenum. Further, following grafts of adult skin and brain the spleen and liver were significantly heavier. Following liver grafts the liver and heart showed a significant increase in weight. Adult chicken spleen grafts caused a marked increase in the weight of the spleen and heart. Further, it was observed

that, regardless of what tissue was used for grafting, 9 days later the morphological integrity of the graft was destroyed and the area was replaced by a myeloid metaplastic centre. On the other hand, embryonic duodena retained their integrity and continued to differentiate.

2. Grafting of adult duodenum caused acceleration of tissue differentiation of the host duodenum. The polysaccharides in the connective tissue and goblet cells of the duodenum differentiated at least 24 hours earlier than in control chicks. Following grafting of duodenum, spleen, and skin, the host spleen exhibited a marked increase in granuloblasts and granulocytes. The heart and liver following grafts were essentially like those in control embryos.

3. Treatment of the adult duodenum prior to grafting with either 95 per cent. alcohol for 24 hours at —20° C., lyophilization, or heating at 80° C. for 20 minutes, resulted in inactivation, the host not being affected.

4. When duodena of 15-, 16-, 17-, 18-, and 20-day embryos were grafted to the CAM, no changes in the weight of host spleen, liver, or heart were found. Following 20-day duodenal grafts the weight of the duodenum was significantly decreased.

5. All embryos receiving adult duodenum, liver, and heart extracts into the yolk sac at 4 days of incubation, died within 72 hours after inoculation.

6. The results are discussed in the light of organ-specific growth stimulation and graft-*versus*-host reaction, both of which fail to adequately explain all the results observed.

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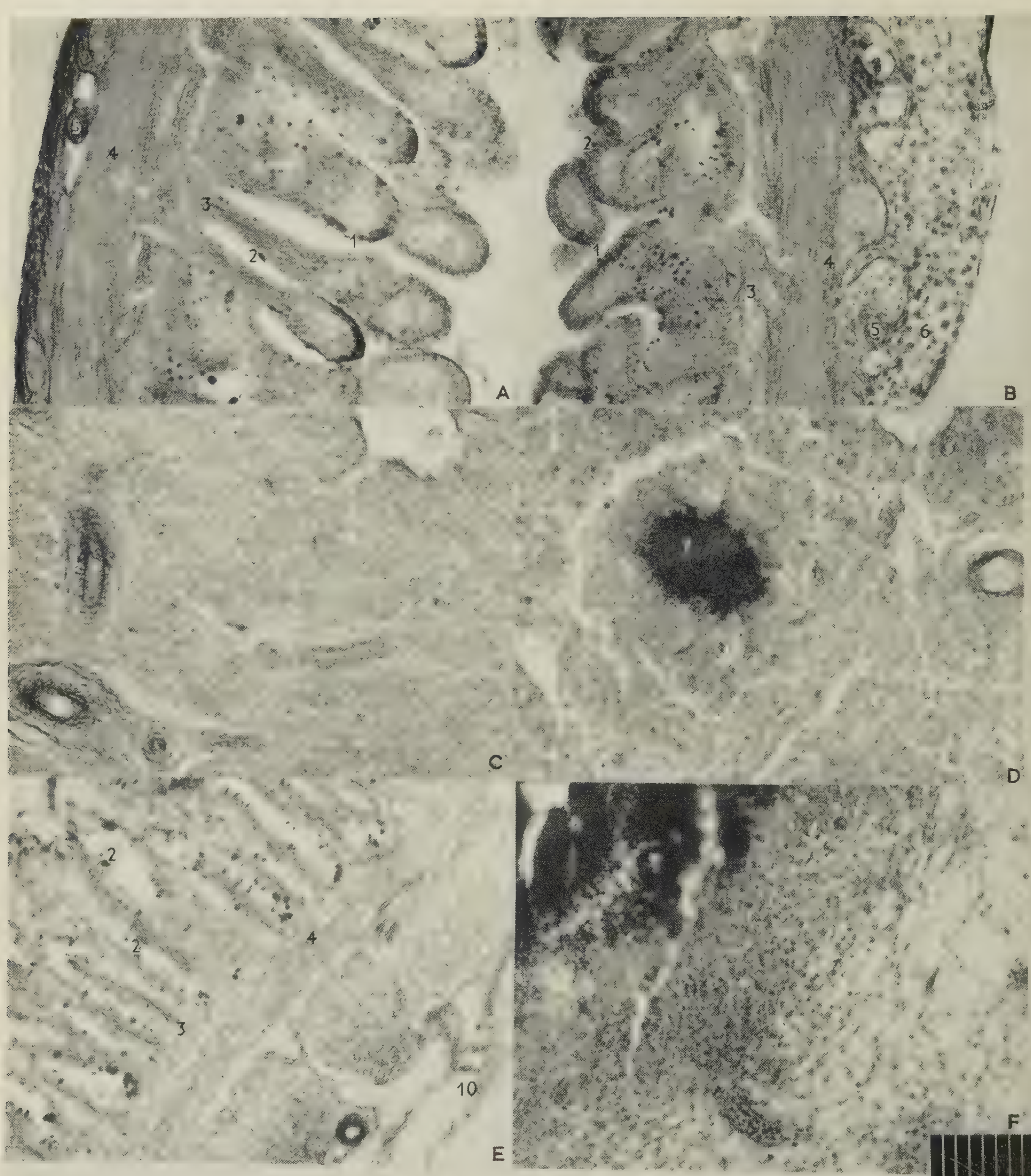

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EXPLANATION OF PLATE

FIG. A. Photomicrograph of a cross-section through the duodenum of a control 18-day embryo. 1. epithelium; 2. goblet cell; 3. lamina propria; 4. tunica muscularis; 5. blood-vessel.

FIG. B. Photomicrograph of a cross-section through the duodenum of an 18-day embryo following adult chicken duodenal grafts. 1. epithelium; 2. goblet cell; 3. lamina propria; 4. tunica muscularis; 5. blood-vessel; 6. lymphocytes.

FIG. C. Photomicrograph of a cross-section through a spleen of a control 18-day embryo showing the reticular structure.

FIG. D. Photomicrograph of a cross-section through a spleen of an 18-day embryo following adult duodenal grafts. 7. granulocytic nodule surrounded by multinucleated giant cells.

FIG. E. Photomicrograph of a cross-section of the CAM through a 15-day embryonic duodenal graft on the 18th day of incubation. 2. goblet cell; 3. lamina propria; 4. tunica muscularis; 10. chorioallantoic membrane.

FIG. F. Photomicrograph of a cross-section of the CAM through an adult duodenal graft on the 18th day of incubation. 8. foci of degenerating adult duodenal tissue surrounded by invading lymphocytes and granulocytes.

All sections were stained by the Himes Moriber triple stain.
Micrometer scale insert: 1 division = 0·01 mm.

(Manuscript received 23 : ii : 59)

The Effects of Chorioallantoic Grafts on the developing Chick Embryo

II. Studies of Adult Antigens in the Duodenum and Spleen

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WITH ONE PLATE

INTRODUCTION

THERE is abundant evidence that the eggs and developing embryos of the chick possess antigenically active materials; that during development changes occur in the antigenic pattern; and that many of these antigens are similar to certain adult antigens. An extensive review and summary of the early literature on the origin of adult antigens in the developing embryo has been made by Needham (1931), Cooper (1946), and Schechtman (1947). Consistent results have been obtained only in recent years by the use of more refined techniques and have been reviewed by Woerdeman (1953), Tyler (1955, 1957), Brachet (1957), and Ebert (1958).

Burke, Sullivan, Petersen, & Weed (1944) prepared antisera against saline extracts of adult organs (brain, testis, ovary, kidney, liver, and lens) of the chicken. They observed that adult organ-specific antigens in the chick embryo appeared subsequent to differentiation and development of the organ, e.g. lens at 146 hours, erythrocytes at 100 hours, kidney at 220 hours, and brain, testis, and ovary at 260 hours. On the other hand, Schechtman (1948) and Ebert (1950) maintained that antigens of the brain, heart, and spleen were identifiable in the early chick blastoderm. Furthermore, adult lens antigens were identified in the embryo at 58 hours by Ten Cate & Van Doorenmaalen (1950) and at the time of lens formation by Flickinger, Levi, & Smith (1955). Recently, Van Doorenmaalen (1958), using the fluorescent antiserum technique, was able to demonstrate specific localization of the marginal zone of the lens epithelium on the 5th day of development. This whole problem of immunochemical study of the development of the lens has been thoroughly reviewed by Ebert (1958).

Schechtman (1948) was the first to identify an organ antigen in the early chick

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embryo, i.e. at the primitive-streak, early neurula, and 4- to 5-somite stages. The ontogeny of tissue antigens in the developing chicken spleen was demonstrated by Ebert (1952). He prepared antisera against saline extracts of 18-day embryonic chick spleen and tested it with saline extracts of 12- and 18-day embryonic spleen. It was observed that the number of antigens increased from three to six between the 12th and 18th day of incubation. Absorption of anti-18-day spleen sera with 12-day embryonic spleen reduced antigenic reactions from three to zero with 12-day spleen antigens and decreased antigenic reactions with 18-day preparations from six to three.

Proteins of the blood-serum of chicks have been identified in the egg-yolk and in extracts of various ages of chick embryos by immunochemical methods (Schechtman, 1955). Antigenic activity of the yolk was reduced by absorption with anti-adult serum, anti-albumin, and anti-γ-globulin (Schechtman, 1947; Nace & Schechtman, 1948). Similar results were obtained by Schechtman & Hoffman (1952) using anti-$\alpha\beta$-globulin serum. Nace (1953) observed the ontogeny of blood proteins in the course of development. He was able to demonstrate that serum of chick embryos contained a vitelloid albumin present from the 3rd day, a non-vitelloid albumin by the 5th day, a vitelloid γ-globulin by the 9th day, and a non-vitelloid γ-globulin by the 12th day; a non-vitelloid $\alpha\beta$-globulin was present from the 6th day.

From the above literature it appears that it may be possible to detect an ontogeny of adult antigens in the formation of the duodenum which might be correlated with previous studies of morphological and chemical differentiation as carried out by Moog (1950), Richardson, Berkowitz, & Moog (1955), and Van Alten & Fennell (1957). It was also believed that this would be fundamental to a study of the effect of chorioallantoic grafts on the antigenic pattern of the duodenum. The details of this study were given by Van Alten & Fennell (1959).

MATERIALS AND METHODS

A modification of the agar diffusion technique of Ouchterlony (1949), which has recently been reviewed by Oudin (1952), was used in the study of adult antigen formation in the embryonic duodenum (11 to 21 days of incubation). The geometry of the plates has been described by Fox & Yoon (1958).

Pooled adult White Leghorn chicken duodena and large numbers of embryonic chick duodena of 11 to 21 days of incubation were used as a source of antigens, the pancreas having been removed. When antigenic preparations were needed for immunization or for serological tests, the adult tissue was homogenized in a Waring blendor for 10 minutes, followed by a second homogenization with a glass homogenizer for 30 minutes at 4° C. in saline (0·15 M NaCl + 0·005 M phosphate, pH 7·4), in a ratio of 1 g. wet weight of tissue per 5 ml. of saline. The homogenate was centrifuged at 4° C. for 30 minutes at 500 g. Antigens of embryonic chick duodena of 11 to 21 days were prepared in a similar manner except that all homogenizing was done with an all-glass homogenizer. Protein determination of the antigens was made by the biuret method of Lowry, Rosebrough, Farr, & Randall (1951). The antigen prepared from adult duodenum was found to contain 19 mg. of protein per ml. and the antigens prepared from embryonic duodena contained about 6 mg. of protein per ml. in each preparation.

Antisera were prepared against adult chicken duodenum and spleen by injecting the pooled antigen into three male rabbits. Five intravenous injections were given during a 10-day period, one every other day. The first two injections were 0·5 ml. of antigen each; the third was 1 ml., the fourth 1·5 ml., and the fifth 2 ml. The rabbits were rested for 2 weeks following the injections and then given an anamnestic injection of 2 ml. of antigen intravenously. Seven days later the rabbits were bled and a precipitin titration was made. If the titre was at least 1:64 the rabbit was bled on the following day and the sera were collected; 1:5,000 merthiolate was added, complement inactivated by heating at 56° C. for 30 minutes and the sera frozen until used. If the titre was not 1:64, the rabbit was given another 2-ml. injection of antigen on the following day and the serum titrated after another 8 days. This procedure was carried out until a sufficient titre was obtained.

RESULTS

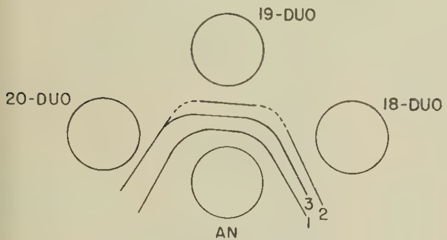
The appearance of adult duodenal antigens in the developing embryonic duodenum of the chick

Antisera against saline extracts of adult chicken duodenum were tested with saline extracts of adult and 11- to 21-day embryonic chick duodena by a modification of the methods of Ouchterlony (1949) and Bjorklund (1952).

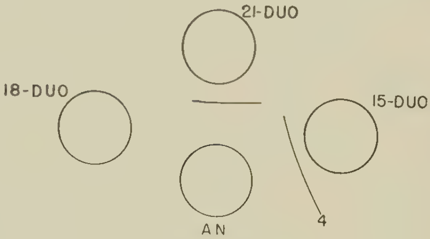
Plate, fig. A shows a diagram of an Ouchterlony plate obtained with anti-adult duodenum serum. This plate consisted of agar with four wells; the lower well contained the antiserum while the other three wells contained saline extracts of 12-, 13-, and 14-day embryonic duodenum. In the plate a series of lines of precipitate have developed where there was optimum concentration of antigens and antiserum, and these have been numbered 1, 2, and 3 in the diagram.

The results of these experiments show that there were changes in the number of antigens present in the duodenum of the chick embryo between 11 and 21 days of incubation. From the 11th to the 13th day only two lines were formed. On the 14th day a third line was present (line 3, Plate, figs. A, B). Line 4 appeared on the 15th day and was also found on the 16th and 17th days (Plate, figs. C, D). However, on the 18th day only lines 1, 2, and 3 were present and line 4 was absent (Text-fig. 1). Lines 1, 2, and 3 remained up to the 21st day, and a fourth line was also present at this stage, but it is not possible from the data to ascertain if this line was identical to line 4 in 15-day duodena.

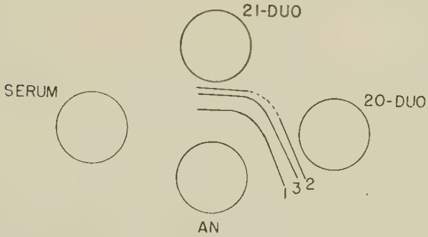
The distribution of line 4 has been confirmed by repetition of experiments and also by means of the Bjorklund inhibition technique. When the latter technique was used results were obtained which are shown in Text-fig. 2. In this plate six doses of 0·15 M saline extract of 18-day embryonic duodenum were put into the antiserum well prior to putting the antigens and antiserum into their respective wells. It was shown in Text-fig. 1 that 18-day duodenal antigen reacted with antiserum to form lines 1, 2, and 3. Following the inhibition procedure lines 1, 2, and 3 were absent but line 4 developed adjacent to the 15-day well (Text-fig. 2).



TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.

The presence of line 4 and also the line adjacent to the 21-day well suggests that these antigens may be qualitatively distinct from those found in the 18-day duodenum.

The results presented in Text-fig. 3 were obtained in essentially the same manner as those in Text-fig. 2, with the exception that adult chicken serum was used as the inhibiting antigen. The position of lines 1, 2, and 3 adjacent to 20- and 21-day wells is comparable to those in Text-fig. 1. This suggests that lines 1, 2, and 3 are not serum antigens. The absence of the line adjacent to the 21-day well, as illustrated in Text-fig. 2, suggests that this antigen has reactive groupings in common with serum.

Table 1 summarizes the distribution of duodenal antigens during the period from 11 to 21 days. The inhibition analyses are also summarized. It has been demonstrated that (1) the pattern of adult duodenal antigens found in the developing duodenum changes during development, and (2) at least three of the antigens found in the embryonic duodenum do not appear to be present in adult serum.

TABLE 1

A summary of the results obtained with the serum-agar precipitin test

Adult duodenum antiserum was tested with saline extract of adult and 11- to 21-day chick embryonic duodena

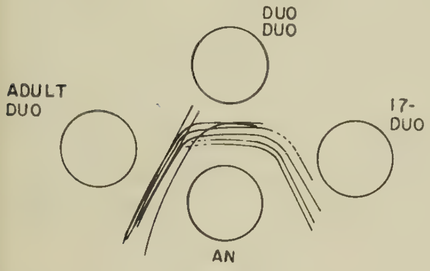
Antiserum	*Antigen*	*No. lines*	*Inhibition*	*No. lines*
Anti-adult duodenum	11-day embryo duodenum	2	..	..
,, ,,	12-day embryo duodenum	2	..	..
,, ,,	13-day embryo duodenum	2	..	..
,, ,,	14-day embryo duodenum	3	..	..
,, ,,	15-day embryo duodenum	4	18-day embryo duodenum	1
,, ,,	16-day embryo duodenum	4	..	..
,, ,,	17-day embryo duodenum	4	..	..
,, ,,	18-day embryo duodenum	3	18-day embryo duodenum	0
,, ,,	19-day embryo duodenum	3	..	..
,, ,,	20-day embryo duodenum	3	Adult serum	3
,, ,,	21-day embryo duodenum	4	Adult serum	3
,, ,,	21-day embryo duodenum	4	18-day embryo duodenum	1
,, ,,	Adult duodenum	9	..	..
,, ,,	Adult serum	3	Adult serum	0

The effect of chorioallantoic transplants of adult chicken duodenum and spleen on the development of adult duodenal and splenic antigens in the duodenum and spleen of 17-day embryos

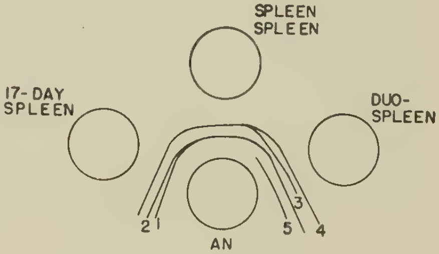
Antisera against saline extracts of pooled adult chicken duodena or against adult chicken spleen were tested with saline extracts of (1) normal 17-day embryonic chick duodenum or spleen; (2) 17-day embryonic duodenum or spleen which was stimulated by CAM (chorioallantoic membrane) grafts of adult chicken duodenum; (3) 17-day embryonic duodenum or spleen which was stimulated by CAM grafts of adult chicken spleen. Immunochemical methods similar to those mentioned in the preceding section were used.

The antigenic pattern of the developing duodenum following CAM grafts of adult chicken duodenum or spleen is shown in Plate, figs. E, F. In this figure the antiserum well contained anti-adult chicken duodenum and the other wells contained antigenic mixtures prepared from 17-day embryonic duodenum which had been stimulated by grafts of adult spleen (Spleen Duo), antigenic mixtures prepared from 17-day embryonic duodenum which had been stimulated by grafts of adult duodenum (Duo-Duo) and antigenic mixtures prepared from control 17-day embryonic duodenum (17-Duo). It may be seen that opposite wells 17-Duo, Duo-Duo, and Spleen Duo, lines 1 to 4 are present. This demonstrates the presence of an antigenic component of identical specificity in all three preparations. It will be further noted that line 5 is only opposite wells Duo-Duo and Spleen Duo. On the other hand, lines 6 and 7 are present only opposite well Duo-Duo.

Text-fig. 4 shows an Ouchterlony plate obtained with anti-adult chicken duodenum serum and tested with antigenic mixtures prepared from adult duodenum (Adult Duo), antigenic mixtures prepared from 17-day embryonic duodenum which had been stimulated by adult duodenum grafts (Duo-Duo), and antigenic mixtures prepared from control 17-day embryonic duodenum (17-Duo). It was evident (see Text-fig. 4; Plate, figs. E, F) that grafting of adult duodenum to the CAM of 9-day chick embryos caused an increase in antigenic components 4 to 7 in the 17-day embryonic chick duodenum. It was also observed that if adult chicken spleen was transplanted to the CAM, one extra antigenic component was found, but this one was also found if duodenum was used on the CAM.



TEXT-FIG. 4.



TEXT-FIG. 5.

It has been shown by Van Alten & Fennell (1959) that the spleen of the embryo undergoes a marked increase in size subsequent to grafting adult duodenum or adult spleen to the CAM. Text-fig. 5 shows an Ouchterlony plate obtained with anti-adult chicken spleen in the antiserum well and saline soluble antigenic mixtures prepared from control 17-day embryonic spleen (17-day Spleen), antigenic mixtures prepared from 17-day embryonic spleen stimulated by adult spleen grafts (Spleen Spleen) and antigenic mixtures prepared from 17-day embryonic spleen stimulated by adult duodenum grafts (Duo-Spleen) in the other 3 wells. Lines 1, 2, and 4 can be seen opposite well 17-day Spleen, and from other plates it has been confirmed that line 4 consists of two antigenic components which are seen as lines 3 and 4 opposite the Duo-Spleen well. Only two lines can be seen opposite well Spleen Spleen, but these have also been shown to consist of two lines each; it can therefore be concluded that lines 1, 2, 3, and 4 are all present. Lines 3 and 4 are clearly seen opposite well Duo-Spleen. However, lines 1 and 2 form one line as they did opposite well Spleen Spleen. It is clear then that lines 1 through 4 are opposite each of the antigen wells and are continuous, and that they therefore demonstrate antigenic components of identical specificity. However, because the lines are together opposite some of the wells and divided when opposite others, this shows that there are quantitative differences in the various spleens. Line 5, on the other hand, is present only opposite well Duo-Spleen, thus clearly demonstrating a qualitative difference.

From the above results it can be seen that grafting of adult chicken duodenum to the CAM of 9-day chick embryos causes both quantitative and qualitative antigenic differences in the duodenum and spleen of 17-day embryos. On the other hand, grafting of adult spleen to the CAM produces both qualitative and quantitative changes in the antigenic picture of 17-day duodenum, but only quantitative changes were observed in the host spleens.

DISCUSSION

It was observed in this study that antigens of the adult chicken duodenum can be identified in the 11-day embryonic duodenum. However, from the 11th to the 21st day of incubation there is an increase in the number of antigens in the developing duodenum; thus, two antigens were present in 11-, 12-, and 13-day embryos; three antigens in 14-day embryos; four antigens in 15-, 16- and 17-day embryos; three antigens in 18-, 19-, and 20-day embryos; and four antigens in 21-day embryos. Ebert (1952) identified an increase in the number of antigens in the development of the chick spleen. He observed three antigens in spleens of 12-day embryos and six in spleens of 18-day embryos when he used 18-day embryonic spleen antiserum.

The appearance of an antigen on the 15th day and its disappearance on the 18th day is rather striking. A somewhat similar situation has been reported by Telfer & Williams (1953) in the *Cecropia* silk worm. They identified five pupal blood antigens present throughout metamorphosis, with a sixth appearing at the fifth instar. The latter antigen persisted during the pupal stage and disappeared in the adult. In the case reported in this study the antigen observed on the 15th day was also found in the adult because antiserum was prepared against adult duodenum. It must be kept in mind that at the present time it is not possible to prove the complete absence of an antigen on the basis of its absence in agar plates.

Several explanations for the appearance and disappearance of an antigen during the course of development can be advanced. Tyler (1957) suggested that many of the large molecular substances of an organism may have similar determinant groups and therefore may react with the same antiserum. Thus, it might be that during the 15th, 16th, and 17th day the fourth antigen which was present may not have been the actual adult antigen, but rather one which had similar determinant groups and thus reacted with the antiserum. This disappearance, then, could be ascribed to an alteration of determinant groups in the course of development, so that a reaction with the antiserum no longer occurred. However, a more satisfactory explanation would seem to be one related to changes which might occur in protein configuration during morphogenesis.

During morphogenesis there may be a structural change in antigenic macromolecules. Fox (1958) suggested that an antigen may be transformed from a complete antigen into an incomplete antigen. An incomplete antigen is one which has specificity for antibody formation but which will not react in the precipitin test. A somewhat different change in antigenic structure could occur, i.e. a change from a globular to a fibrous protein. Mazia & Dan (1952) observed that during spindle fibre formation there was a change from globular to fibrous protein. Heidelberger (1956) suggests that antigenic specificity may be due to multiple reactive areas on the surface of antigens. He believes that changes in specificity of native proteins can be accounted for by the unfolding of polypeptide chains which leads to their separation from adjoining chains, and that, as a consequence, the specificity of the antigen is altered.

If Heidelberger's concept correctly explains antigenic specificity it is reasonable to assume that during the course of development of an antigen its polypeptides may undergo foldings and separation. Under these conditions the reactive surface of the molecule is altered and cannot be detected by immunological methods. On the basis of the evidence on hand and the wide divergence of views of antigen reactivity, a final explanation cannot be given as to why line 4 appears in 15-day duodena and disappears in 18-day duodena.

It has been further observed in the course of this study that transplantation of adult chicken organs (duodenum and spleen) to the CAM caused changes in the antigenic pattern of both the duodenum and spleen. This change was most pronounced following grafting of adult duodenum. Adult duodenal grafts increased the number of antigens from 4 to 7 in the 17-day embryonic duodenum and from 4 to 5 in the spleen. The grafting of adult spleen altered the antigenic pattern of the 17-day duodenum (from 4 to 5) but was without effect in the spleen. It must be kept in mind, however, that even these changes in the antigenic picture are the end products of differentiation and not the causal factors.

Ebert (1955) maintained that growth of host tissues following homologous grafts was due to a 'building block' rather than a 'template' or catalytic mechanism (Weiss, 1947). The findings in this investigation tend to support this 'building block' hypothesis, i.e. selective incorporation of tissue-specific antigens from grafts into homologous host tissues. However, this hypothesis does not adequately explain the generalized effects, i.e. growth of heterologous organs. Also, the 'building block' hypothesis does not allow for the possibility that the graft may be exerting an influence which causes an acceleration of antigenic differentiation rather than a direct transfer mechanism. The evidence of this investigation does not rule out the possibility of a catalytic mechanism as advanced by Weiss (1947). This catalytic mechanism could be similar to that of increasing the substrate and accelerating differentiation of a specific enzyme. Recently, Kato & Moog (1958) have shown that after injection of disodium phenylphosphate into the chick on the 14th day of incubation there was an increased alkaline phosphatase activity in the duodenum, liver, mesonephros, and metanephros on the 17th day of incubation.

This study has shown that when adult duodenum or spleen were grafted the increase in the number of antigens was not confined to the homologous organ.

It might be postulated that some non-specific material, common to several organs, might accelerate growth and enhance differentiation of the adult antigenic pattern.

SUMMARY

1. The development of adult duodenal antigens in the embryonic duodenum was studied by means of the Ouchterlony agar diffusion technique.

2. In 11-, 12-, and 13-day embryonic duodena there were two antigens; by the 14th day three antigens; in the 15-, 16-, and 17-day duodena four antigens; while in 18-, 19-, and 20-day duodena there were only three antigens with a fourth antigen again present in the 21-day duodenum. The disappearance of the fourth antigen found in 15-, 16-, and 17-day duodena was attributed to a change in molecular configuration of the antigen in the process of differentiation.

3. Following grafts of adult duodenum there was an increase of three antigens observed in the host duodenum and an increase of one antigen in the host spleen.

4. When adult spleen was grafted there was no increase in the number of antigens in the host spleen, but an increase of one antigen was observed in the duodenum.

5. The results are discussed in the light of the organ-specific growth stimulation hypothesis, but this is rejected because it cannot account for the generalized effects observed.

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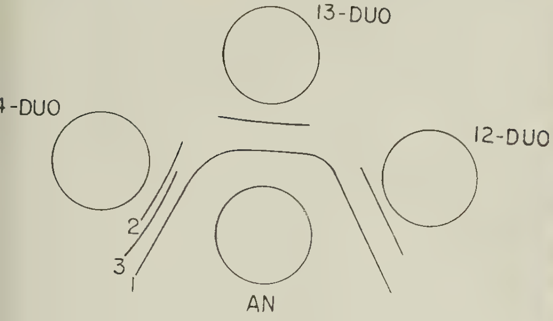

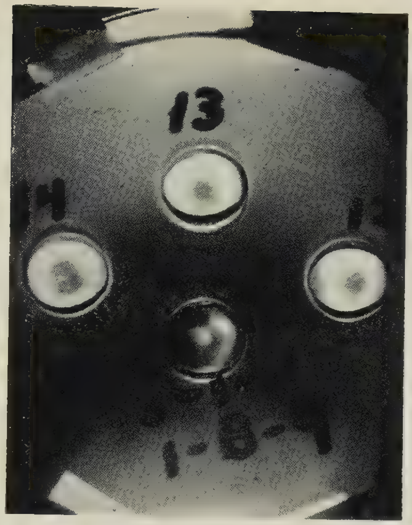

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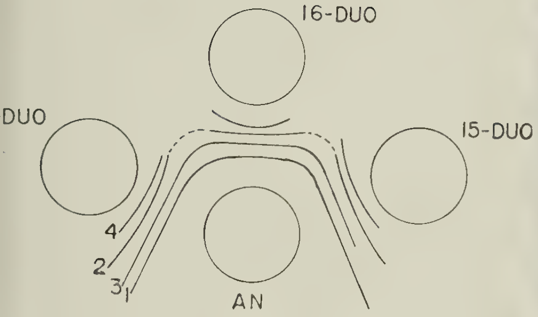

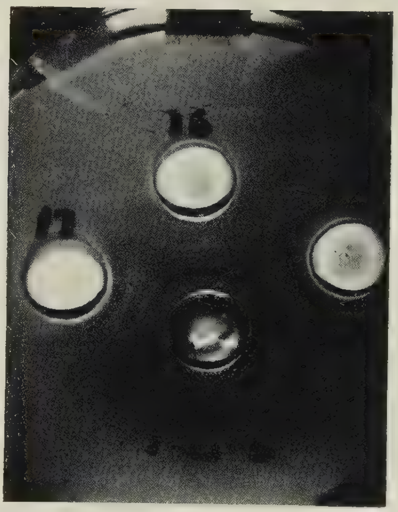

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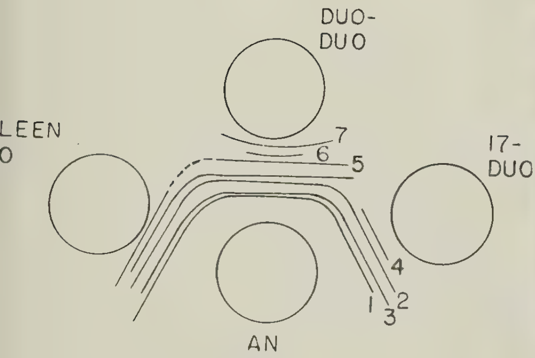

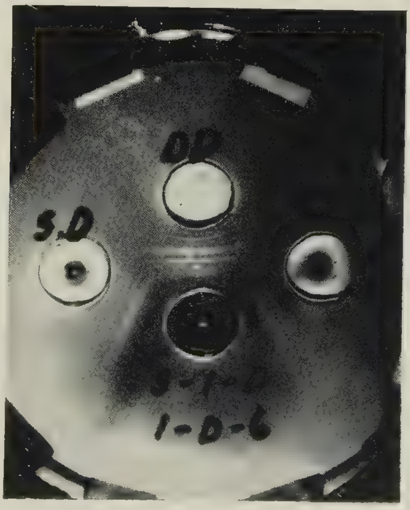

F

P. J. VAN ALTEN

Le facteur 'temps' dans l'action inductrice du chordo-mésoblaste et l'âge de l'ectoblaste réagissant

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AVEC DEUX PLANCHES

INTRODUCTION

LES recherches expérimentales de Nieuwkoop (1952), de Eyal-Giladi (1954), de Sala (1955) et de Johnen (1956), pour ne citer que les contributions les plus importantes, semblent prouver irréfutablement que le chordo-mésoblaste tout entier possède un certain pouvoir d'induire des structures prosencéphaliques dans l'ectoblaste jeune et compétent. En partant de ces résultats expérimentaux Nieuwkoop a élaboré une théorie de l'induction selon laquelle la formation du cerveau antérieur correspondrait aux tendances évolutives intrinsèques à l'ectoblaste tout entier; l'action inductrice de la voûte archentérique se limiterait à déclencher, ou à activer, comme le dit Nieuwkoop, ces tendances primitivement latentes. La phase d'activation précéderait inévitablement le développement ultérieur des régions plus caudales, notoméritiques, de l'ébauche neurale. Une action relativement tardive, dite transformatrice, exercée par le chordo-mésoblaste amènerait l'ectoblaste auparavant activé, et par conséquent déjà orienté vers la formation du prosencéphale, à constituer le rhombencéphale et la moelle.

Il convient d'insister sur le fait que le processus de l'induction neurogène a été étudié par Nieuwkoop et ses collaborateurs dans des conditions expérimentales s'écartant considérablement des normales, la technique employée étant, le plus souvent, celle des 'sandwiches' ectoblastiques. Des fragments d'inducteur prélevés sur de jeunes neurulas ont été placés entre deux lambeaux d'ectoblaste excisé d'embryons au début de la gastrulation. Ces 'sandwiches' ont été cultivés *in vitro* dans la solution saline de Holtfreter. Cette méthode a été adoptée dans le but de pouvoir étudier le processus de l'induction dans un milieu aussi 'neutre' que possible. On peut se demander, pourtant, si le développement des structures neurales induites dans des 'sandwiches' ectoblastiques n'est pas altéré encore plus profondément par ce procédé expérimental qu'il ne l'est dans le cas de la transplantation sur la face ventrale d'un embryon. En fait, le développement de tels 'sandwiches' est toujours retardé considérablement par rapport au développe-

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ment d'embryons-témoins et, d'autre part, la zone marginale dans les vésicules neurales constituées par ces explantats est fréquemment plus ou moins déficiente (Gallera, 1948; Woelwarth, 1952; Nieuwkoop & Nigtevecht, 1954; Denis, 1957). Qui plus est, Barth (1941) a constaté que chez l'*Amblystoma punctatum* une exposition de la face interne de l'ectoblaste explanté à l'action de la solution saline suffit déjà pour provoquer la formation des structures neurales acrencéphaliques dans cet ectoblaste. Quoique chez d'autres espèces d'Urodèles le feuillet externe soit moins sensible, le même résultat a pu être obtenu avec de l'ectoblaste isolé de gastrulas d'Axolotl et de Triton et cultivé dans des solutions salines à tendances légèrement cytolytiques (Holtfreter, 1944–7; Karasaki, 1957). Certes, dans des 'sandwiches' la face interne de l'ectoblaste est plus ou moins protégée contre l'action directe de la solution saline; cependant Nieuwkoop & Nigtevecht (1954), Hori & Nieuwkoop (1955) et Sala (1956) ont observé la formation de petits complexes acrencéphaliques aux bords de leurs 'sandwiches' préparés aux dépens de l'ectoblaste d'Axolotl. Ces 'activations locales', comme les appellent les auteurs cités, ont été tout à fait indépendantes des structures induites par des fragments de la voûte archentérique enrobés dans l'ectoblaste. Il paraît donc légitime de supposer que la culture de l'ectoblaste isolé dans une solution saline puisse dans une certaine mesure orienter le développement de cet ectoblaste vers la formation des structures acrencéphaliques. (Rappelons enfin que Ter Horst (1948), en cultivant des fragments de la voûte archentérique dans des 'sandwiches' ectoblastiques, a observé, dans quelques cas, la neuralisation directe et apparemment paradoxale du matériel chordo-mésoblastique.)

Il faut remarquer, d'autre part, que dans les expériences mentionnées cidessus, l'ectoblaste très jeune a été soumis à l'action d'un inducteur beaucoup plus âgé. Or, comme nous le savons, grâce aux recherches de Okada & Takaya (1942), de Okada & Hama (1943, 1945), de Takaya (1953) et de Hoessels (1957), la nature même du pouvoir inducteur du matériel préchordal présomptif change complètement au cours de la gastrulation. Quoique le chordo-mésoblaste ne manifeste pas de telles inversions de son pouvoir inducteur, la possibilité de modifications d'une moindre ampleur doit être toujours prise en considération.

Cette mise en relief du caractère un peu artificiel des expériences de Nieuwkoop et de ses collaborateurs s'impose d'autant plus que la théorie de l'induction neurogène élaborée par cet auteur ne tient pas, à mon avis, suffisamment compte des résultats des autres recherches expérimentales, entreprises dans des conditions plus proches de celles réalisées au cours du développement normal. En effet, si la formation de l'acrencéphale dans l'ectoblaste une fois activé était autonome et soumise à la loi du 'tout ou rien' comment pourrait-on expliquer les réductions quantitatives et qualitatives du prosencéphale, réductions qu'on obtient facilement en excisant le substratum inducteur même aux stades relativement avancés du développement? Certes, le parachèvement du développement de l'acrencéphale, en particulier la bilatéralité des yeux, serait dû selon Nieuwkoop & Nigtevecht (1954) à une action transformatrice exercée tardivement par la plaque préchordale elle-même. Cependant, les arguments à l'appui de cette thèse, d'ailleurs à peine esquissés par ces auteurs, ne me semblent pas convaincants. D'autre part, les investigations de Waddington & Deuchar (1952), ainsi que les miennes (1951, 1958), ont montré que le neurectoblaste postérieur, déjà orienté par une action suffisamment longue du chordo-mésoblaste vers la formation du rhombencéphale ou de la moelle, peut encore, après avoir été mis expérimentalement en contact avec la préchorde, fournir un cerveau antérieur plus ou moins rudimentaire. Il serait superflu d'insister sur le fait que ce résultat est tout à fait incompréhensible dans le cadre de la théorie de Nieuwkoop. Enfin, si cette théorie était vraie, il faudrait s'attendre à ce que le matériel notoneurique présomptif (ectoblaste destiné à bâtir le rhombencéphale et la moelle) isolé suffisamment tôt de son substratum inducteur se développe en acrencéphale. Les possibilités évolutives de cet ectoblaste, soumis à l'action inductrice pendant un temps plus ou moins court, ont été examinées par Damas (1947), Eyal-Giladi (1954) et Denis (1957). Malheureusement, ces expériences n'ont pas apporté de solution à notre problème. Damas a exécuté de nombreuses transplantations de neurectoblaste médullaire présomptif sur la face ventrale de jeunes neurulas. Les greffons ont été prélevés sur des embryons aux stades de la gastrulation et au début de la neurulation. Les expériences de Denis ont été, du point de vue technique, plus compliquées: un tronçon de la plaque médullaire d'une neurula a été remplacé par un fragment de l'ectoblaste beaucoup plus jeune, prélevé au pôle animal d'une gastrula. Ce greffon, après avoir été laissé pendant un temps variable sur la région troncale antérieure de la voûte archentérique de son hôte, a été repris et transplanté sur la face ventrale d'une autre neurula. (Dans un certain nombre d'expériences, les greffons, enrobés dans l'ectoblaste provenant de jeunes gastrulas, ont été cultivés *in vitro*, dans une solution saline. Cependant, cette méthode d'élevage en 'sandwich' s'est révélée inopportune.) Les résultats obtenus par ces deux auteurs sont les mêmes dans leurs grandes lignes: les greffons ont donné des tissus variés: épiderme, cellules ganglionnaires, migratrices et pigmentaires, masses neurales compactes ou creuses; pourtant, la formation d'organes, pas plus de moelle que de structures cérébrales ou sensorielles, n'a jamais été observée. Cependant, tandis que Damas insiste sur le fait que ses greffons ont fourni des dérivés d'autant plus complexes qu'ils ont été prélevés sur des embryons plus âgés, les résultats obtenus par Denis n'offrent pas une telle régularité. Certains de ses implantats jeunes n'ont donné que de l'épiderme atypique, tandis que d'autres, ayant subi une action inductrice de même durée, se sont transformés en tissu nerveux parfaitement différencié. Dans quelques cas ce dernier résultat a été obtenu déjà après 3 heures de contact inducteur. Denis en a conclu qu'une très faible impulsion inductrice de départ suffit déjà pour que l'ectoblaste évolue en tissu nerveux. Cette évolution se poursuivrait d'une façon autonome, bien que les conditions d'élevage peu favorables soient encore capables de la dévier vers la production des autres tissus, tels que l'épiderme et les dérivés de la crête neurale. La gradation décrite par certains auteurs dans l'induction neurogène ne serait qu'illusoire et due à des conditions d'élevage défavorables et difficiles à contrôler.

A ce propos quelques commentaires me semblent indispensables. Certes, l'arrêt plus ou moins complet de la morphogenèse de l'ébauche neurale n'exclut pas nécessairement la possibilité d'une cytodifférenciation neuronale parfaite. Cependant, ce serait simplifier le problème à l'excès, si on voulait en conclure que les processus histogénétiques et morphogénétiques sont indépendants les uns par rapport aux autres. En particulier la cytodifférenciation régionale du cerveau est en large mesure tributaire de l'accomplissement normal de la morphogenèse de cette ébauche; et j'ai apporté moi-même quelques arguments à l'appui de cette thèse dans mes travaux antérieurs (1955, 1956). D'autre part l'individualisation des diverses catégories d'éléments cellulaires que fournit le neurectoblaste au cours de son évolution est sûrement liée causalement à l'élaboration de différentes substances cytospécifiques. Déjà du point de vue théorique on devrait s'attendre à ce que le principe des seuils différentiels, correspondant à ces diverses catégories cellulaires, trouve ici sa pleine application. En effet, Denis a constaté qu'au point de vue statistique trois phases principales (épiderme, dérivés de la crête neurale, tissu nerveux) ont pu être distinguées dans la série des prestations de ses greffons ordonnés suivant la durée de plus en plus longue de leur contact avec l'inducteur. La neuralisation de l'ectoblaste exposé à l'action inductrice pendant un laps de temps très court n'a été observée que dans quelques cas rares, où des implantations un peu spéciales ont empêché l'étirement du greffon sur le ventre de son hôte. Dans un travail récent, Denis (1958) a constaté que l'inertie cinématique de ces greffons aboutissait à leur neuralisation. Au lieu de transplanter ses greffons sur la face ventrale de neurulas, il a pratiqué leur insertion dans le blastocoele de jeunes gastrulas. Dans ces conditions, l'étirement des greffons a été empêché et, quoique soumis à une brève induction, ils ont fourni un nombre appréciable de structures neurales. Denis en conclut que l'étirement du greffon inhibe la neuralisation déclenchée déjà par un bref contact inducteur. Les recherches de Ter Horst (1948), de Töndury (1948) et de Dalcq & Lallier (1948) ont montré que le blocage des processus cinématiques propres au matériel chordo-mésoblastique peut le conduire à la neuralisation directe (sans intervention d'un inducteur quelconque). Se référant à cette conclusion, ne serait-il pas indiqué d'envisager la proposition inverse à celle proposée par Denis, à savoir que précisément le freinage des mouvements morphogénétiques inciterait l'ectoblaste à la neuralisation et renforcerait l'action inductrice insuffisante. Rappelons encore qu'au cours du développement normal la plaque neurale subit une forte extension dans le sens cranio-caudal. Quoique le mécanisme de l'étirement d'un greffon sur la face ventrale de son hôte ne soit pas identique à celui de l'extension de l'ectoblaste dorsal, il me semble évident que cet étirement, aussi atypique qu'il soit s'écarte pourtant moins des conditions normales que le tassement du matériel greffé défectueusement ou que l'inertie cinématique des greffons implantés dans le blastocoele.

Les expériences de Mme Eyal-Giladi (1954) ont été faites à l'aide d'une technique très particulière, empruntée à Nieuwkoop. Des bandes ectoblastiques transversales, excisées de la région médullo-rhombencéphalique présomptive de jeunes embryons, ont été pliées en deux et, après la soudure des deux faces internes de l'ectoblaste, les greffons ont été enfoncés par l'une de leurs extrémités dans une incision faite à la face ventrale de l'embryon-hôte. Ce procédé, permettant de réduire la zone de contact entre le greffon et son hôte au strict minimum, a été adopté afin d'éliminer dans la mesure du possible l'influence éventuelle exercée par l'embryon sur l'implantat. Les greffons de Eyal-Giladi ont souvent fourni, contrairement aux résultats obtenus par Damas et Denis, de l'acrencéphale typique. Cependant, selon cet auteur, ces structures prosencéphaliques ont été induites non par l'ébauche chordale et somitique mais par la plaque préchordale glissant sous l'ectoblaste durant la gastrulation. (Il faut remarquer que l'*Amblystoma mexicanum*, espèce utilisée par Eyal-Giladi, se caractérise par une grande rapidité du processus inducteur.) Un contact un peu plus prolongé avec le chordo-mésoblaste suffisait déjà pour 'transformer' ces tendances acrencéphaliques en tendances à former du rhombencéphale ou de la moelle.

Étant données les opinions contradictoires, relatées ci-dessus, au sujet du rôle joué par le substratum inducteur dans la formation de l'ébauche neurale et compte tenu, d'autre part, des résultats souvent équivoques de divers essais de vérification expérimentale, j'ai cru utile de reprendre l'analyse de l'induction du rhombencéphale. On admet généralement que ce processus est en principe de même nature que celui de l'induction de la moelle épinière. Cependant, cette dernière n'offre pas des critériums et des indices morphologiques suffisants pour permettre un diagnostic sûr dans les cas où les greffons fournissent, comme cela arrive fréquemment, des structures neurales plus ou moins difformes. Dans la première série de mes expériences le neurectoblaste sera soustrait plus ou moins tôt à l'action inductrice de son substratum normal; dans la deuxième série, l'ectoblaste très jeune, prélevé sur une gastrula, sera pendant un temps variable mis en contact avec un inducteur beaucoup plus âgé. La confrontation des résultats de ces deux séries d'expériences permettra de contrôler si les capacités inductrices du chordo-mésoblaste évolué sont toujours les mêmes qu'au moment où ce matériel atteignait, au cours de la gastrulation, la région de l'ectoblaste correspondant au rhombencéphale futur.

MATÉRIEL ET TECHNIQUE

Toutes les expériences ont été faites sur les embryons de *Triturus alpestris*; elles se répartissent en deux séries principales et quelques expériences de contrôle.

Les embryons ont été opérés aseptiquement sur le fond d'agar dans la solution de Holtfreter additionnée d'un sulfamidé (Elkosine Ciba) dans la proportion de un pour mille. Les expériences de la première série consistaient en transplantations des fragments de neurectoblaste présomptif sur la face ventrale de jeunes neurulas. Les donneurs ont été colorés au bleu de Nil. Les prélèvements du matériel destiné à être transplanté ont été faits aux stades successifs de la gastrulation, du stade 'grand blastopore circulaire', correspondant à la phase initiale de l'induction, au début de la neurulation. Un intervalle approximativement de 20 heures s'écoule entre ces deux stades, comme l'atteste mes observations sur des embryons-témoins. Les greffons, en forme de larges bandes transversales, contenaient toujours le même matériel embryonnaire, à savoir le

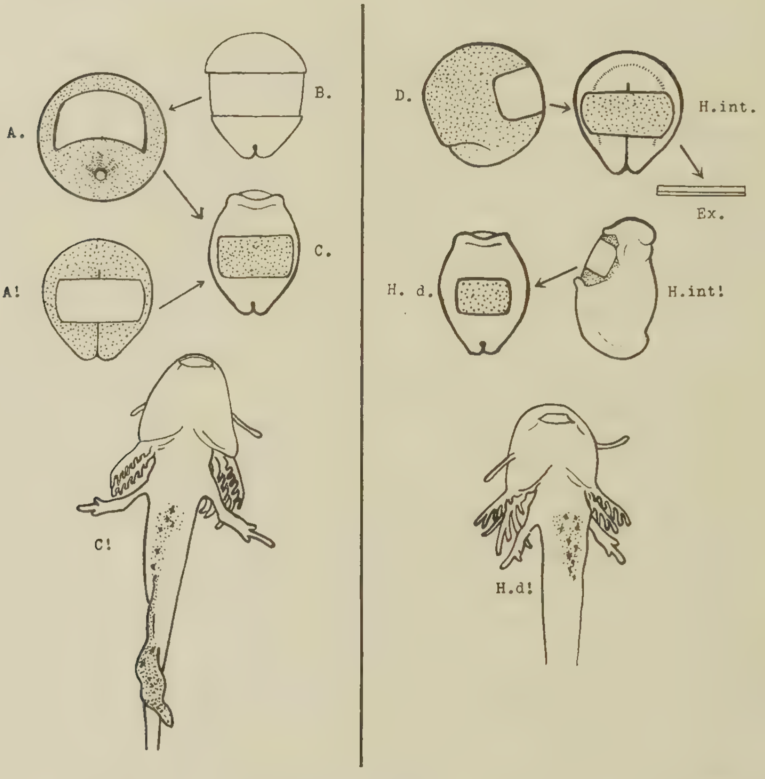

FIG. 1. Schéma des opérations: à gauche — expériences de la première série; à droite — deuxième série. A. et A.', donneurs (gastrula au petit blastopore circulaire et jeune neurula). B., donneur supplémentaire (jeune neurula vue du côté ventral). C., hôte au moment de transplantation. C.', hôte au moment de fixation. D., donneur (vu de profil). *H. int.*, hôte intermédiaire au moment de transplantation. *H. int.'*, hôte intermédiaire au moment de fixation. *Ex.*, explantat. *H.d.*, hôte définitif au moment de transplantation. *H.d.'*, hôte définitif au moment de fixation. Embryons et greffons colorés au bleu de Nil — pointillés.

rhombencéphale présomptif presque entier et une petite portion du neurectoblaste médullaire; les bords latéraux des greffons ont été formés d'épiblaste (voir Fig. 1, à gauche). Pour faciliter la localisation exacte du matériel prélevé sur des gastrulas, la blessure faite dans le feuillet externe de ces dernières a été comblée par un lambeau d'épiblaste ventral prélevé sur de jeunes neurulas (donneurs supplémentaires). Ces greffons âgés et incapables de différenciation neurale ont été toujours bien visibles sur les donneurs principaux, même plusieurs jours après l'opération. Seuls ces greffons neurectoblastiques, dont la localisation était strictement telle qu'on l'avait voulue, ont été pris en considération dans ce travail.

Dans la deuxième série d'expériences (Fig. 1, à droite), de larges bandes d'ectoblaste antéro-ventral, prélevé sur des gastrulas munies d'un blastopore en fer à cheval ou d'un grand blastopore circulaire, ont été transplantées sur la voûte archentérique de jeunes neurulas (hôtes intermédiaires). Les donneurs ont été colorés, comme d'habitude, au bleu de Nil. Les greffons ont été enchâssés dans une large lacune créée préalablement dans le neurectoblaste rhombencéphalique et médullaire présomptif des hôtes intermédiaires. Le greffon de neurectoblaste a été ensuite excisé et sa face interne a été couverte d'un lambeau d'épiblaste ventral prélevé sur une autre neurula; les explantats ainsi constitués ont été cultivés dans la solution de Holtfreter. Après un laps de temps s'étendant de 10 à 28 heures selon le cas, les greffons ont été excisés de nouveau et transplantés sur la face ventrale d'autres neurulas, cette fois les hôtes définitifs.

Les embryons-porteurs des greffons et les explantats, après avoir été transportés dans la solution de Holtfreter fraîche, ont été élevés pendant 11 à 19 jours; puis ils ont été fixés au Bouin et examinés sur les coupes sériées de 8 μ d'épaisseur (coloration à l'hémalun et le mélange d'éosine et d'orange). Les embryons et les explantats ont été élevés à la température du laboratoire. La période entre l'apparition de l'encoche blastoporale et la formation du sillon médian était approximativement de 24 heures; environ 8 heures plus tard les bourrelets médullaires devenaient déjà bien visibles.

Il faut remarquer que mes donneurs de la deuxième série d'expériences ont été opérés approximativement à l'âge où le chordo-mésoblaste atteint au cours de la gastrulation le niveau du deutomérite. L'ectoblaste transplanté a été donc mis en contact avec l'inducteur au moment de son évolution où le même phénomène se produit au cours du développement normal; par contre, l'âge du substratum inducteur des hôtes intermédiaires a été sensiblement plus avancé, exactement comme c'était le cas dans les expériences de Denis et dans les 'sandwiches' de Nieuwkoop et de ses collaborateurs. Je dois encore dire d'avance que l'examen histologique de mes explantats rhombencéphalo-médullaires a apporté un appoint précieux aux résultats de mes expériences de la première série.

RÉSULTATS EXPÉRIMENTAUX

Série I. Transplantation des fragments neurectoblastiques prélevés sur des embryons aux stades successifs de la gastrulation et au début de la neurulation

Les greffons, en suivant l'allongement général du tronc de leurs hôtes, s'étirent considérablement dans le sens cranio-caudal et prennent la forme de longues bandes bleues s'étendant, dans la majorité des cas, du niveau des membres antérieurs jusqu'à la région anale des larves qui les portent. Les greffons les plus jeunes, et même quelques-uns de plus âgés, demeurent tout le temps en surface et s'incorporent harmonieusement à l'épiderme de l'hôte dont ils ne se distinguent plus que par leur coloration bleue. Cependant quelques-uns de ces greffons jeunes ont fourni encore des mélanophores bien visibles à travers l'épiderme coloré au bleu de Nil. Ces cellules pigmentaires ont apparu vers la fin de la première semaine d'élevage ou au début de la semaine suivante. Les greffons plus âgés ont donné, outre de l'épiderme et des cellules pigmentaires, des structures neurales plus ou moins distinguables déjà à l'examen de leurs hôtes *in vivo*. Quelques-uns de ces greffons se sont enfoncés, semble-t-il, entièrement, sans fournir de l'épiderme, et ils ont été recouverts secondairement par l'épiblaste de l'hôte. L'apparition des bourrelets médullaires sur le neurectoblaste implanté a été fréquemment observée; mais dans la plupart des cas, ces bourrelets se sont bientôt effacés complètement et ce n'est que dans quelques cas exceptionnels que la formation d'une gouttière neurale, d'ailleurs toujours trop étroite, et sa fermeture en tube, suivant le processus habituel, a pu être constatée. Il appert donc que la neuralisation de greffons ectoblastiques s'accomplit le plus souvent d'une façon atypique, décrite par Denis: le fragment implanté, ou sa portion principale, se ramasse sur lui-même, s'enfonce, puis il est recouvert par l'épiderme ambiant et, enfin, une cavité centrale se creuse au sein de cette masse neurale primitivement compacte.

Notons encore que les greffons âgés ont fréquemment donné naissance aux petites 'nageoires dorsales', situées évidemment sur la face ventrale des larves. Dans plusieurs cas, l'épiderme de ces nageoires était nettement coloré par le bleu de Nil, ce qui prouve qu'elles se sont constituées entièrement aux dépens du matériel greffé. Les nageoires en question formaient toujours l'extrémité postérieure du greffon.

Les résultats de l'examen histologique de mes greffons sont rapportés globalement sur le tableau 1. Comme on le voit, un certain nombre de greffons prélevés au cours de la gastrulation n'ont formé que de l'épiblaste incorporé dans celui de l'hôte. Cet épiblaste issu de greffons était pourtant plus épais et pourvu de nombreuses cellules glandulaires. Dans quelques cas une petite portion du matériel greffé s'est différenciée en crête neurale dispersée en éléments pigmentaires et ecto-mésenchymateux (Planche 1, fig. A). La plupart des greffons prélevés sur des gastrulas âgées (à partir du stade de petit blastopore circulaire) ont donné des structures neurales dont le caractère morphologique, cérébral, médullaire ou ganglionnaire, n'a pas pu souvent être précisé. Par contre, les greffons les plus âgés, prélevés au début de la neurulation, ont fourni tous, à l'exception d'un seul, du rhombencéphale dûment reconnaissable, dans quelques cas même parfaitement conformé (Planche 1, fig. B) et accompagné de petits otocystes. Ces rhombencéphales se continuaient vers l'arrière soit dans un cordon neural de caractère indéfini, souvent très long et mince, soit, quoique plus rarement, dans une moelle typique. De même que mes greffons âgés, tous les explantats excisés de jeunes neurulas ont donné du rhombencéphale plus ou moins normal et accompagné, dans la majorité des cas, des ganglions et des vésicules auditives (Planche 1, fig. C). (Les résultats de ces dernières expériences sont résumés en bas du tableau 1 (*b*).) Notons encore que les structures neurales, fournies soit par

TABLEAU 1

(*a*) *Transplantation du matériel rhombencéphalique et médullaire présomptif sur la face ventrale de jeunes neurulas*

	Stade du donneur au moment de l'opération	*Grand blastopore circulaire*	*Blastopore circulaire moyen*	*Petit blastopore circulaire*	*Petit blastopore allongé*	*Début de la neurulation*
	Nombre d'embryons opérés	4	7	13	4	13
Nombre de cas où les greffons ont donné	éléments de la crête neurale	2	5	9	4	13
	structure neurale indéfinie		4	6	1	3
	rhombencéphale			1	2	12
	otocystes			1	1	4
	ganglions			1		1
	moelle				1	5
	nageoire dorsale		1	4	3	6
	Nombre de cas d'induction de muscles		1	3		4

(*b*) *Explantations du matériel rhombencéphalique et médullaire*

	Stade de l'embryon au moment de l'opération	*Sillon médian*	*Liséré pigmenté*	*Bourrelets médullaires*
	Nombre d'explantats	1	6	8
Nombre d'expl. qui ont donné	crête neurale	1	6	8
	rhombencéphale	1	6	8
	otocystes	1	4	5
	ganglions	1	5	4
	muscles		1	5

les greffons soit par les explantats, ont été toujours accompagnées par des cellules ecto-mésenchymateuses et pigmentaires plus ou moins nombreuses selon le cas.

Parmi les structures neurales produites aux dépens des greffons jeunes, prélevés sur des gastrulas, on peut distinguer toutes les formes de transition entre un amas de cellules ganglionnaires et une vésicule neurale. Ce sont soit des nodules composés de fibres au centre, et de cellules à la périphérie; soit des masses neurales creusées de quelques petites cavités (si ces dernières sont situées à la périphérie, elles sont fermées par une sorte de toile choroïdienne); soit, enfin, des vésicules neurales de forme irrégulière dont la nature rhombencéphalique a pu, pourtant, être reconnue dans quelques cas grâce à la présence d'une (Planche 1, fig. D et E) ou, plus rarement, des deux cellules de Mauthner. Il faut encore remarquer qu'indépendamment du degré de développement réalisé par les structures neurales énumérées ci-dessus, la limitante gliale externe était toujours absente, les cellules neurales étaient souvent assemblées assez lâchement et les fibres nerveuses situées à la périphérie se dispersaient librement dans le milieu ambiant ou, dans d'autres cas, étaient groupées en cordons se dirigeant vers l'intestin de l'hôte. Ce fait explique le phénomène apparemment insolite que j'ai pourtant observé maintes fois, à savoir la présence des cellules pigmentaires arborisées au sein des formations neurales issues de mes greffons. Comme nous le savons grâce aux investigations de Twitty & Niu (1948) et de Székely (1953), les cellules pigmentaires, dérivées de la crête neurale, se repoussent mutuellement et sont attirées par le tissu nerveux. (Les chromatophores sécréteraient une substance diffusible et absorbée activement par le tissu nerveux; d'autre part, les mouvements de ces cellules pigmentaires seraient toujours orientés suivant le décrément de concentration de substances sécrétées par elles-mêmes.) Rien d'étonnant alors qu'en absence de la barrière habituelle constituée par la limitante gliale, les chromatophores, toujours abondants au voisinage des formations neurales issues de mes greffons, puissent se glisser entre les cellules neurales relâchées et pénétrer secondairement à l'intérieur de la substance blanche primitive (Planche 1, fig. F).

Des nageoires 'dorsales' supplémentaires ont été fréquemment observées sur le ventre de mes larves; elles étaient situées en arrière des structures neurales bâties par les greffons. La formation de ces nageoires, due en premier lieu à l'abondance d'ecto-mésenchyme, a été sûrement facilitée par l'influence régionale exercée par le segment tronco-caudal des hôtes.

Il reste encore à mentionner que dans de nombreux cas le tractus digestif de l'hôte a induit dans l'ecto-mésenchyme issu des greffons la formation de petits cartilages; en revanche, les structures neurales médullaires bâties par les greffons ont provoqué dans quelques cas la différenciation de cellules de somatopleure en fibres musculaires striées. Rappelons qu'une telle action de la moelle sur le mésoblaste latéral et ventral a été observée chez les Urodèles par Detwiler & Holtzer (1956).

La présence de fibres musculaires, d'ailleurs toujours peu nombreuses, a été constatée dans plusieurs de mes explantats, particulièrement dans les plus âgés. Il est évident que ces fibres se sont différenciées à partir de quelques cellules du substratum que j'ai laissées par mégarde adhérer à la face interne de mes explantats. En effet, ces explantats ont été faits au cours d'interventions opératoires de la série suivante d'expériences, de sorte que j'ai été forcé de les laisser un certain temps dans la solution de Holtfreter avant de nettoyer leur face interne. Or, d'une part, le matériel chordo-mésoblastique adhère très intimement à la plaque neurale, et d'autre part, les fragments neurectoblastiques s'enroulent presque instantanément, ce qui rend leur nettoyage très difficile si on ne le fait tout de suite après l'excision.

En récapitulant les résultats des expériences relatées ci-dessus, je dois insister sur le fait que mes greffons n'ont jamais fourni des structures acrencéphaliques. La première manifestation de l'action inductrice (que les greffons ont subi avant leur transplantation) consistait toujours dans la formation de la crête neurale dispersée en cellules pigmentaires et ecto-mésenchymateuses; les greffons plus âgés ont été capables de fournir des amas ganglionnaires, éventuellement des vésicules neurales de taille réduite et de forme irrégulière. La nature déjà rhombencéphalique de quelques-unes de ces vésicules a été attestée par la présence des cellules de Mauthner. La formation du rhombencéphale et de la moelle typiques n'a été observée qu'à partir des greffons prélevés au début de la neurulation.

Quant à l'influence régionale exercée éventuellement par les tissus adjacents des hôtes sur le développement de mes greffons, elle ne s'est pas manifestée clairement dans mes expériences; il est pourtant probable que la formation des nageoires supplémentaires, situées dans la région troncale postérieure des larves, a été 'facilitée' par ce facteur. Il est significatif que les structures cérébrales fournies par les greffons âgés ne diffèrent pas, à une exception près, de celles formées par les explantats d'âge correspondant. Dans ces derniers le rhombencéphale est généralement accompagné de beaux ganglions, lesquels font, le plus souvent, défaut dans les greffons. Il se peut donc que dans l'ambiance embryonnaire de l'hôte les cellules ganglionnaires manifestent une certaine tendance à se disperser.

Denis, au cours de ses expériences, dont j'ai esquissé l'analyse sommaire dans l'introduction, n'a observé que sporadiquement la formation des structures neurales aux dépens des fragments de l'ectoblaste soumis pendant un certain temps à l'action inductrice et cultivés ultérieurement entre deux lambeaux ectoblastiques dans la solution de Holtfreter. Ces structures neurales ont été toujours imparfaites, en particulier la formation de fibres nerveuses n'a jamais été observée, même après deux semaines de culture *in vitro*. Au contraire, tous mes explantats rhombencéphaliques excisés de jeunes neurulas de Triton ont donné du cerveau postérieur plus ou moins bien conformé et accompagné souvent des ganglions et des vésicules auditives, induits dans le lambeau épiblastique accolé à la face interne de mes explantats. La différenciation histologique de ces complexes cérébraux a été le plus souvent parfaite, bien qu'un peu retardée par rapport à la norme; en particulier la digestion du vitellus a été très lente. (Les cellules nerveuses du rhombencéphale dont la coupe transversale est représentée sur la fig. C de la Planche 1, étaient très chargées de plaquettes vitellines; malheureusement celles-ci, vu leur coloration en jaune clair, ne sont pas visibles sur la microphotographie.) La différence entre les résultats de Denis et les miens ne peut être attribuée au fait que nous avons utilisé deux espèces différentes, à savoir *Pleurodeles waltii* et *Triturus alpestris.* En effet, dans mes expériences antérieures (1955) j'ai obtenu la formation du cerveau entier et des organes des sens lui correspondant à partir de la plaque cérébrale excisée de très jeunes neurulas de Pleurodèle. L'examen histologique de mes préparations, imprégnées à l'argent, a révélé que les grandes commissures cérébrales et les principales voies nerveuses se sont formées, quoique le nombre total des fibres nerveuses ait été plus restreint que dans un cerveau normal. On est donc amené à supposer que la cytodifférenciation défectueuse des explantats préparés par Denis est due à la technique employée par cet auteur, technique que j'ai utilisée moi-même dans mes expériences de 1948. Ces expériences m'ont donné, du point de vue de la différenciation histologique, de mauvais résultats; et depuis lors je cultive ou bien les explantats nus (1955) ou bien, comme je l'ai fait dans mes expériences actuelles, seule la face interne des explantats est couverte d'un lambeau épiblastique. Denis, en utilisant mon ancienne technique, a cultivé des fragments de l'ectoblaste dans des sacs épiblastiques contenant, certes, la solution de Holtfreter, mais déjà profondément modifiée grâce au métabolisme cellulaire, et dont la constitution était semblable à celle du liquide remplissant le blastocèle d'embryons normaux (Stableford, 1949). Il paraît donc que l'exposition de la face externe de l'ectoblaste à l'action d'un milieu extra-embryonnaire normal (eau, solution de Holtfreter) soit indispensable pour que cet ectoblaste puisse poursuivre sa différenciation nerveuse normale.

Série II. Action inductrice du matériel chordo-somitique d'une neurula sur l'ectoblaste jeune, prélevé sur une gastrula

Rappelons que dans cette série d'expériences de grands greffons, en forme de larges bandes transversales, ont été excisés de l'ectoblaste antéro-ventral de gastrulas munies d'un blastopore circulaire ou en fer à cheval. Les greffons ont été reportés sur le dos de jeunes neurulas où ils ont été enchâssés dans une blessure de forme et dimensions correspondantes pratiquée dans le neurectoblaste d'embryons-hôtes (voir Fig. 1, à gauche, p. 492). Le matériel neurectoblastique enlevé aux hôtes contenait la portion rhombencéphalique et, partiellement, la région médullaire antérieure de la plaque neurale. Les greffons sont laissés un laps de temps varié sur la voûte archentérique des hôtes et, ensuite, ils sont retransplantés sur la face ventrale d'autres neurulas, qui sont, cette fois, leurs hôtes définitifs.

L'observation des hôtes intermédiaires a révélé que les greffons restaient apparemment inertes au moins pendant 17 heures; cependant ils subissaient une certaine extension en longueur et leurs bords antérieurs et postérieurs ont pris la forme arquée. Au moment voulu, des rectangles, les plus grands possible, ont été excisés de ces greffons pour être transplantés sur la face ventrale d'autres neurulas. Les greffons définitifs étaient, par conséquent, un peu plus petits et contenaient, dans la majorité des cas, seulement le matériel rhombencéphalique présomptif.

TABLEAU 2

Prestations des greffons ectoblastiques prélevés sur des gastrulas, reportés sur la région rhombencéphalique-médullaire de jeunes neurulas, et, après un certain laps de temps, retransplantés sur la face ventrale d'autres neurulas

Nombre de cas où le greffon a été laissé sur le substratum inducteur		*de 10 à 16 h.* 10	*de 16 à 22 h.* 18	*de 22 à 28 h.* 11
Nombre de cas où les greffons ont donné	éléments de la crête neurale	1	14	10
	acrencéphale	1	5	
	œil		3	
	placode olfactive		3	
	structure neurale indéfinie		6	8
	rhombencéphale		3	2
	otocyste		1	
	ganglions		1	2
	moelle		1	4?
	muscles		4	
Nombre de cas d'induction de muscles			2	

Les larves-porteuses de ces greffons se distinguaient déjà par leur aspect extérieur de celles de la série précédente. La formation de nageoires supplémentaires n'a jamais été observée; les greffons, moins allongés que dans la série précédente, formaient des bosses ovoïdes situées généralement entre les membres antérieurs des hôtes.

Les résultats de cette série d'expériences sont réunis dans le tableau 2 par ordre de durée de l'action inductrice que les greffons ont subi sur les hôtes intermédiaires. La différence de 18 heures entre les durées maximum et minimum de contact inducteur a été divisée par trois, et le tableau a été partagé en colonnes verticales correspondant aux intervalles envisagés. Les greffons qui n'ont formé que de l'épiderme n'ont pas été reportés sur le tableau. Comme on le voit, c'étaient surtout les greffons jeunes, rangés dans la première colonne, qui ont donné ce résultat; cependant l'un des greffons âgés (troisième colonne) a aussi évolué en surface, en se transformant tout entier en épiderme. (Il se peut que par une faute d'implantation passée inaperçue le contact entre ce greffon et le chordo-mésoblaste de son hôte intermédiaire n'ait pas été suffisamment étroit.) Les 24 greffons (nombre total 39) ont fourni des structures neurales plus ou moins développées: nodules composés de cellules et fibres nerveuses; vésicules cérébrales de forme irrégulière et de caractère indéfini, rhombencéphales typiques, un court tronçon médullaire; dans 6 cas, enfin, des complexes neuro-sensoriels de caractère indéniablement acrencéphalique se sont constitués. La formation du cerveau antérieur aux dépens de mes greffons, phénomène jamais observé dans la série précédente d'expériences, mérite un examen détaillé. A l'exception d'un seul cas où le greffon a été laissé à peine 10 heures et 15 minutes sur le substratum inducteur d'un hôte intermédiaire, dans tous les autres cas l'acrencéphale a été bâti par des greffons d'âge moyen, soumis préalablement à l'action inductrice durant 18 à 22 heures. Abstraction faite de cellules pigmentaires et ecto-mésenchymateuses toujours présentes, quatre greffons n'ont donné que de l'acrencéphale et, éventuellement, des organes des sens lui correspondant. Deux greffons se sont développés en cerveau entier. Le greffon le plus jeune, soumis préalablement à l'action inductrice pendant 10 heures, a formé une petite vésicule prosencéphalique dont le plancher était épais et la voûte mince; cette dernière a donné naissance à une paraphyse typique. Dans un autre cas l'acrencéphale a été réduit à un petit nodule neural; cependant sa nature prosencéphalique ne peut être mise en doute puisqu'il a été relié par un cordon fibreux à un œil petit mais parfaitement constitué (Planche 2, fig. G). Les autres greffons en question ont bâti le cerveau antérieur de forme irrégulière et de taille plus ou moins réduite. Dans deux cas le caractère prosencéphalique de ces structures neurales n'est attesté que par la présence de belles placodes olfactives. Dans un autre cas, le greffon a donné un acrencéphale relativement bien constitué. En effet, le diencéphale et le télencéphale se sont individualisés et la voûte de ce dernier a formé une paraphyse, tandis que le plancher s'est différencié en bulbe olfactif relié par un nerf à une petite placode (Planche 2, fig. H). Un greffon, enfin, le plus âgé, a formé un cerveau entier assez bien conformé, à l'exception pourtant du télencéphale qui était tout à fait rudimentaire. Le diencéphale a été muni d'une belle épiphyse et il a donné naissance à deux yeux petits mais typiquement constitués. (Cas unique, dans mes expériences, de bilatéralité de l'appareil oculaire; voir la figure I de la Planche 2.) Le rhombencéphale était accompagné d'une vésicule et d'une placode auditives.

Quant aux autres structures neurales fournies par mes greffons, elles étaient, dans leurs grandes lignes, les mêmes que celles obtenues dans la première série d'expériences. Les greffons d'âge moyen (deuxième colonne du tableau 2) n'ont en général fourni que des formations plutôt neuroïdales que neurales. C'étaient des amas de cellules nerveuses dont la différenciation cytologique était le plus souvent parfaite, mais qui semblaient être dépourvues complètement des capacités organo-formatrices. Dans quelques cas les couches cellulaires les plus superficielles de ces amas se sont décollées sur une certaine étendue de la masse centrale en formant des membranes épithéliales minces; dans d'autres cas de ce genre, les greffons se présentaient sous l'aspect d'une masse amorphe de fibres et cellules nerveuses au sein de laquelle se sont creusées de petites cavités. Cependant l'une de ces structures d'apparence neuroïdale a pu être reconnue, grâce à la présence d'une belle cellule de Mauthner, comme un rhombencéphale rudimentaire.

Les formations neurales bâties par mes greffons ont été toujours accompagnées de cellules ecto-mésenchymateuses et pigmentaires, et dans un certain nombre de cas, de même que dans la première série d'expériences, quelques-unes de ces dernières cellules ont pénétré à l'intérieur des structures neurales en question (Planche 1, fig. F).

Notons encore que, dans un cas exceptionnel, le greffon s'est transformé en un court tronçon médullaire, donnant encore un ganglion situé au-dessous de la plaque basale de cette moelle rudimentaire (Planche 2, fig. J). Je dois pourtant remarquer que ce greffon est l'un des quatre qui ont fourni, outre les formations neurales et les dérivés habituels de la crête neurale, de petits amas musculaires constitués sans doute par les cellules mésoblastiques des hôtes intermédiaires prélevées par mégarde avec les fragments neurectoblastiques (voir le tableau 2, avant-dernière rangée horizontale). C'est à la présence d'un tel fragment du substratum inducteur que doit être, probablement, imputé le caractère nettement spinal des prestations fournies par le greffon en question.

Il reste encore à mentionner que les greffons les plus âgés (troisième colonne sur le tableau 2) ont formé, dans la plupart des cas, de volumineuses vésicules neurales au plancher et parois latérales épaisses, tandis que leur voûte était souvent très mince, de l'aspect d'une toile choroïdienne. Cependant, vu la forme fréquemment très irrégulière de ces vésicules, ce n'est que dans deux cas que des rhombencéphales plus ou moins bien conformés ont pu être identifiés.

Dans la première série de mes expériences, quelques-uns des greffons très jeunes se présentaient sous la forme d'un mince et long cordon cellulo-fibreux creusé tout au plus sur une étendue fort limitée d'un étroit canal central. Les greffons plus âgés ont bâti soit du rhombencéphale, soit des vésicules neurales plus ou moins irrégulières, dont l'aspect cérébral postérieur n'a pas pu, pourtant, être mis en doute. Ces structures cérébrales se continuaient vers l'arrière dans des cordons nerveux, tels que ceux formés par les greffons jeunes, ou, plus rarement, dans une moelle assez bien conformée. En revanche, dans la deuxième série d'expériences la formation des structures neurales de caractère plus ou moins spinal n'a été observée que dans quelques cas rares; et toujours encore ces cordons cellulo-fibreux ou tronçons médullaires ont été très courts. Rappelons encore que ce n'est que dans la première série d'expériences que j'ai observé la formation de petites nageoires dorsales supplémentaires. Cependant, le nombre relativement restreint de greffons de la deuxième série d'expériences qui ont fourni des structures de caractère plus ou moins troncal s'explique par le fait que ces derniers greffons contenaient moins de matériel médullaire présomptif que ceux de la première catégorie.

En ce qui concerne une certaine influence inductrice et réciproque exercée par les tissus adjacents de l'hôte et les dérivés du greffon, les relations ont été les mêmes dans les deux séries d'expériences. Comme je l'ai déjà mentionné, dans de nombreux cas l'intestin de l'hôte a induit la formation de petits cartilages dans l'ecto-mésenchyme issu de greffons; et les structures neurales bâties par ces derniers ont provoqué, dans quelques cas, la différenciation de cellules de somatopleure en fibres musculaires striées.

SYNTHÈSE ET DISCUSSION DES RÉSULTATS

La seule différence entre mes deux séries d'expériences est que: dans la première l'inducteur et l'ectoblaste réagissant ont été de même âge, tandis que dans la deuxième série l'ectoblaste jeune, prélevé au moment où l'induction neurogène débute au cours du développement normal, a été mis en contact avec la voûte archentérique de jeunes neurulas (c'est-à-dire avec l'inducteur arrivé déjà à ce stade de son évolution où, dans l'embryon normal, le processus de l'induction du système nerveux est déjà achevé dans ses grandes lignes). Dans ces deux catégories d'expériences l'ectoblaste a été soustrait à l'action inductrice plus ou moins précocement, mais toujours avant que le processus inducteur ne fût complètement achevé.

La seule différence frappante entre les résultats de mes deux séries d'expériences est que l'ectoblaste soumis à l'action inductrice exercée par le matériel chordal et somitique de l'âge lui correspondant n'a jamais fourni des complexes cérébraux et sensoriels acrencéphaliques; tandis que dans la deuxième série d'expériences de telles structures ont été édifiées par un tiers environ des greffons soumis préalablement à l'action inductrice de durée moyenne. Je ne vois d'autre possibilité que d'en conclure qu'au cours des derniers stades de la gastrulation le pouvoir inducteur du chordo-mésoblaste se modifie considérablement. Au début de la neurulation ce matériel acquiert une certaine capacité d'induire dans l'ectoblaste jeune la formation du cerveau antérieur. Certes, une telle capacité, quoique très faible, existe probablement déjà dans le chordo-mésoblaste jeune; en tout cas, pourtant, elle devient beaucoup plus manifeste chez les embryons plus âgés.

Avant d'aborder la discussion détaillée de ce problème, passons en revue les manifestations évolutives communes à mes deux sortes de greffons. Comme nous l'avons vu, l'action inductrice se manifeste tout d'abord par la formation de la crête neurale dispersée en éléments ecto-mésenchymateux et pigmentaires; l'ectoblaste, laissé un peu plus long sur la voûte archentérique, est déjà capable d'évoluer partiellement en cellules nerveuses disposées sans ordre apparent, en masses neurales d'aspect plutôt ganglionnaire que cérébral ou médullaire. Le stade suivant de l'évolution réalisée est la formation des vésicules cérébrales ou, éventuellement, de la moelle épinière. Il est remarquable qu'à ces étapes successives du développement, réalisé par l'ectoblaste soumis à l'action inductrice de plus en plus longue, correspond une progression quantitative nette; et, de ce point de vue, mes observations rejoignent entièrement celles de Denis: plus le contact avec le chordo-mésoblaste a été prolongé, plus grande est la portion du greffon évoluant en crête neurale; ou, si l'action inductrice a été suffisamment longue, plus volumineuses sont les structures neurales induites dans l'ectoblaste greffé. Cette progression quantitative s'explique aisément si on applique le principe des seuils différentiels au processus de la neuralisation de l'ectoblaste. En effet, comme on l'admet couramment, la voûte archentérique induit très tôt dans l'ectoblaste sus-jacent la formation d'un champ-gradient neurogène. La potentialité de différenciation neurale atteint son intensité maximum au centre de ce champ et décroît vers sa périphérie. On peut par la pensée esquisser des lignes d'équivalence entourant concentriquement le centre de ce champ-gradient; et alors certaines de ces lignes doivent correspondre aux seuils de l'intensité suffisante pour déclancher la différenciation de l'ectoblaste soit en crête neurale, soit en tissu nerveux proprement dit. Au fur et à mesure du processus inducteur, la potentialité de différenciation neurale s'accroît en commençant par le centre du champ-gradient neurogène et en progressant vers sa périphérie, de sorte que les territoires de l'ectoblaste limités par des lignes d'équivalence s'étalent de plus en plus: ce qui explique que les dimensions des formations neurales fournies par mes greffons étaient d'autant plus grandes que ces derniers avaient été soumis plus longtemps à l'action inductrice du chordo-mésoblaste. Comme on se le rappelle, les formations neurales ont été toujours entourées d'éléments ecto-mésenchymateux et pigmentaires; ces éléments, issus de la crête neurale, ont été alors fournis par la zone périphérique de mes greffons, zone située au-delà de la ligne d'équivalence correspondant au seuil de la différenciation en tissu nerveux.

En revenant aux différences d'ordre qualitatif entre les prestations de mes greffons jeunes et âgés, rappelons que les capacités formatrices, éveillées dans l'ectoblaste par l'action inductrice exercée sur lui par la région chordale antérieure de la voûte archentérique, s'ordonnent en gamme allant de la formation de la crête neurale jusqu'à la constitution du cerveau et de la moelle. Quelle position dans cette gamme faut-il attribuer à l'induction de l'acrencéphale dans l'ectoblaste jeune mis en contact avec l'inducteur beaucoup plus âgé, comme c'est le cas dans la deuxième série de mes expériences? Il est remarquable que, dans les expériences en question, les greffons d'âge moyen, c'est-à-dire soumis à l'action inductrice du chordo-mésoblaste durant 16 à 22 heures, aient formé soit de l'épiblaste et des éléments de la crête neurale, soit des structures neuroïdales, soit l'acrencéphale, soit, enfin, le rhombencéphale (et, dans un cas, un tronçon du tube médullaire). Les greffons les plus âgés, soumis à l'action inductrice pendant une période dépassant 22 heures, n'ont jamais fourni de l'acrencéphale; en revanche l'un des plus jeunes greffons de cette série d'expériences a formé un cerveau antérieur rudimentaire. Or, les capacités évolutives d'autres greffons du même âge se bornaient à la formation de l'épiderme banal. Force est donc d'admettre que l'induction de l'acrencéphale suit directement celle de la crête neurale et précède la formation du cerveau postérieur. Il faut insister pourtant sur l'idée que cette conclusion n'est valable que dans le cas d'une action inductrice exercée par le chordo-mésoblaste âgé, et déjà évolué, sur l'ectoblaste beaucoup plus jeune. En fait, dans la première série de mes expériences, où l'âge de l'inducteur correspondait exactement à celui de l'ectoblaste réagissant, l'induction de l'acrencéphale n'a jamais été observée. Comme on se le rappelle, j'en ai déduit qu'au cours des derniers stades de la gastrulation la région chordale de la voûte archentérique acquiert un certain pouvoir inducteur acrencéphalogène, ou, éventuellement, le renforce, si elle en était pourvue déjà auparavant. Cette évolution progressive des capacités inductrices du chordo-mésoblaste est donc de la même nature que celle observée par les auteurs japonais dans la plaque préchordale présomptive. Cependant, elle est beaucoup plus lente et nettement plus restreinte dans le chordo-mésoblaste que dans le matériel préchordal. Un fragment d'ectoblaste jeune, laissé suffisamment longtemps sur la région chordo-mésoblastique de la voûte archentérique d'une jeune neurula, se développe toujours en chordencéphale ou, éventuellement, en moelle épinière. Dans le chordo-mésoblaste la puissance inductrice notogénétique est certainement toujours prédominante. Le faible pouvoir inducteur acrencéphalogène acquis par ce matériel embryonnaire tardivement, est le plus élevé au niveau de l'extrémité antérieure de la chorde et décroît, conformément au schéma proposé par Sala (1955), dans la direction caudale.

En admettant que la puissance inductrice chordencéphalogène et médullaire soit toujours prédominante dans le chordo-mésoblaste, comment serait-il possible d'obtenir la formation du cerveau antérieur dans l'ectoblaste mis en contact avec ce matériel embryonnaire? Ce résultat apparemment incompréhensible, et pourtant obtenu fréquemment, peut être expliqué facilement. Des recherches, trop nombreuses et trop variées pour que je puisse les énumérer ici, semblent montrer que l'induction du cerveau antérieur est due à des substances chimiquement plus simples, plus diffusibles et au poids moléculaire moindre que celles responsables de la formation du cerveau postérieur et de la moelle. Ces premières substances agissent probablement sur l'ectoblaste plus rapidement que les secondes. L'interruption de l'action inductrice au moment opportun nous permettrait de dissocier artificiellement les effets de l'action inductrice acrencéphalogène de ceux provoqués par le pouvoir inducteur deutencéphalique dont le chordo-mésoblaste est toujours doté. Dans ces circonstances la formation de l'acrencéphale dans l'ectoblaste soumis à l'action inductrice du chordo-mésoblaste serait d'autant plus facile à réaliser qu'il réagit plus promptement aux stimuli inducteurs. Fort heureusement, la susceptibilité de l'ectoblaste et, par conséquent, la rapidité de l'induction révèlent de grandes différences chez les quatre espèces d'Urodèles utilisées couramment pour l'expérimentation.

Chez *Triturus alpestris* l'induction est particulièrement lente: en effet, presque un tiers de mes greffons soumis pendant plus que 16 heures à l'action inductrice ont été encore incapables d'édifier des structures neurales, même des plus rudimentaires. En me basant sur mes observations personnelles, aussi bien actuelles que plus anciennes, et sur les données glanées dans la littérature embryologique, je crois que, du point de vue de la vitesse décroissante du processus inducteur, les quatre espèces d'Urodèles en question s'ordonnent comme suit: *Amblystoma mexicanum*, *Pleurodeles waltii*, *Triturus vulgaris* et, enfin, *T. alpestris*. Or, il est instructif que Johnen (1956) dans ses expériences, où de l'ectoblaste d'*A. mexicanum* et de *T. vulgaris* a été soumis à l'action inductrice de courte durée exercée par le chordo-mésoblaste prélevé sur de jeunes neurulas, ait obtenu beaucoup plus fréquemment la formation de l'acrencéphale seul chez la première que chez la seconde espèce. Sala (1955) a obtenu de belles structures acrencéphaliques dans ses 'sandwiches' enfermant des fragments du chordo-mésoblaste; rappelons cependant que cet auteur a utilisé pour ses expériences les embryons d'*A. mexicanum* dont l'ectoblaste réagit particulièrement vite aux stimuli inducteurs. En revanche les explantats neurectoblastiques prélevés sur les embryons de *T. alpestris* par Woellwarth (1952) (ces explantats contenaient toute la portion cérébrale présomptive de la plaque neurale et ils ont été excisés aux stades successifs de la gastrulation et de la neurulation) ont fourni aussi bien de l'acrencéphale que du rhombencéphale.

Un autre problème doit encore retenir notre attention: quelles sont les relations entre l'histogenèse et la morphogenèse des formations neurales fournies par mes greffons? Comme nous l'avons vu, la première manifestation de l'action inductrice (que les greffons ont subi avant leur transplantation) consistait toujours dans la formation de la crête neurale dispersée en cellules pigmentaires et ecto-mésenchymateuses. Cependant quelques-uns des greffons à peine plus âgés ont déjà fourni des structures neurales. Or il importe d'insister sur le fait que même dans les cas où ces structures étaient des plus difformes et indéfinissables du point de vue organogénétique, leur cytodifférenciation neuronale était toujours parfaite. Il semble donc que l'ectoblaste soumis à l'action inductrice acquiert la capacité d'une cytodifférenciation neuronale plus ou moins autonome très tôt, avant que l'orientation de sa morphogenèse générale ne soit stabilisée. Cependant, cette supposition n'exclut nullement la possibilité que l'histogenèse de l'ébauche neurale — de même que sa morphogenèse — ne soit un processus progressif qu'on puisse scinder en étapes successives, correspondant à des durées déterminées de l'action inductrice exercée par la voûte archentérique sur l'ectoblaste. En effet, les formations neurales bâties par mes greffons jeunes se présentaient généralement sous la forme d'amas composés de fibres au centre, et de cellules à la périphérie. Souvent aussi des paquets de fibres nerveuses s'échappaient de ces amas neuraux et se dirigeaient vers l'intestin de l'hôte. Si on se rappelle, enfin, que dans quelques cas des cellules pigmentaires, et peut-être satellites, ont pénétré à l'intérieur des amas en question, il serait difficile de ne pas penser que nous ayons affaire à des ganglions nerveux plus ou moins bien organisés. D'autre part, seuls les greffons âgés ont fourni des vésicules cérébrales, dont la cavité ventriculaire a été limitée par une couche de cellules épendymaires typiques; et les parois de ces vésicules ont été bordées de la limitante gliale externe. Or, comme nous le savons, la formation de cellules épendymaires est indispensable pour que la névroglie puisse se développer. Vu une certaine irrégularité dans le degré de l'histogenèse réalisée par mes greffons jeunes et âgés, et, d'autre part, étant donné le nombre relativement restreint de mes expériences, je n'ose pas en tirer de conclusions définitives; néanmoins il me semble que les possibilités d'histogenèse atteintes progressivement par l'ectoblaste au cours de l'induction neurogène s'ordonnent comme suit: ectomésenchyme et cellules pigmentaires, ganglions nerveux, vésicules cérébrales ou tube médullaire, y compris les diverses sortes de neurones et de cellules névrogliques caractéristiques pour ces formations.

Il est intéressant de noter que plusieurs de mes greffons âgés ont constitué, certes, des vésicules cérébrales, mais leur configuration générale était souvent beaucoup moins régulière que celle des formations neurales fournies par les explantats d'âge correspondant. Il n'y a là rien d'étonnant. En effet, dans le cas de transplantation sur la face ventrale de l'embryon, le développement du greffon est soumis à des conditions mécaniques insolites: aux stades jeunes, le greffon se heurte contre le massif vitellin de son hôte; plus tard la formation et le développement ultérieur de divers organes de ce dernier, tels que le foie, le pancréas, l'intestin, peuvent déformer secondairement l'ébauche neurale issue de l'implantat. Néanmoins, dans un certain nombre de cas la nature rhombencéphalique de telles vésicules difformes a pu être reconnue grâce à leur histogenèse régionalement spécifique. Le criterium le plus sûr d'un tel diagnostic était la formation d'une seule ou des deux cellules de Mauthner. Conformément aux résultats expérimentaux de Stefanelli (1950) — cet auteur a constaté que la destinée évolutive de futures cellules de Mauthner ne se stabilise qu'aux stades relativement avancés de la neurulation — mes greffons prélevés à ces stades n'ont fourni que du rhombencéphale pourvu des deux cellules de Mauthner. En revanche, une cellule de Mauthner impaire s'est différenciée dans quelques formations neurales issues des greffons plus jeunes, prélevés, par exemple, au stade d'un petit blastopore circulaire. Les structures neurales en question étaient soit de forme très irrégulière — et alors la position de la cellule de Mauthner semblait être quelconque — soit, au contraire, le rhombencéphale affectait la forme typique d'un cerveau otocéphale, le sillon médian n'étant pas creusé; les deux lames latérales se sont fusionnées en une seule masse dans laquelle la cellule de Mauthner occupait alors une position toujours strictement médiane. Dans quelques cas enfin, les deux cellules de Mauthner se sont quand même différenciées, mais elles étaient très rapprochées l'une de l'autre. Ainsi donc les rhombencéphales bâtis par mes greffons, et les explantats, peuvent être ordonnés en une série continue selon le degré plus ou moins accentué de la réduction morphogénétique dont ils ont été atteints. Cette série est strictement parallèle à celle bien connue des réductions morphogénétiques progressives du cerveau antérieur, réductions maintes fois observées au cours des expériences déjà classiques telles que l'exposition de jeunes embryons à l'action des solutions de LiCl ou les excisions du substratum inducteur aux stades successifs de la gastrulation et de la neurulation. Rappelons que le premier degré de cette réduction est caractérisé par les malformations du télencéphale et de l'organe olfactif (synrhinie-monorhinie); dans le second degré, le prosencéphale tout entier révèle une déficience morphogénétique nette, telle qu'on la connaît chez les cyclopes; le troisième degré correspond à l'anophtalmie et, dans le cas extrême, nous avons affaire à l'absence totale du cerveau antérieur et moyen.

RÉSUMÉ

Des transplantations des fragments du neurectoblaste rhombencéphalique et médullaire antérieur présomptif sur la face ventrale de jeunes neurulas ont été exécutées sur des embryons de *Triturus alpestris*. Ces expériences se répartissent en deux séries principales: dans la première l'inducteur et l'ectoblaste réagissant ont été de même âge, tandis que dans la deuxième série l'ectoblaste jeune, prélevé sur des gastrulas, a été mis en contact avec la région chordale antérieure de la voûte archentérique de jeunes neurulas (c'est-à-dire avec l'inducteur arrivé déjà à ce stade de son évolution où, dans l'embryon normal, le processus de l'induction du système nerveux est déjà achevé dans ses grandes lignes). Dans ces deux séries d'expériences l'ectoblaste a été soustrait à l'action inductrice plus ou moins précocement, mais toujours avant que le processus inducteur ne fût complètement achevé. Les expériences de transplantation ont été complétées par l'étude du développement des explantats rhombencéphaliques excisés de jeunes neurulas.

La première manifestation de l'action inductrice (que les greffons ont subie avant leur transplantation) consistait toujours dans la formation de la crête neurale dispersée en cellules pigmentaires et ecto-mésenchymateuses; les greffons plus âgés ont été capables de fournir des amas ganglionnaires, éventuellement des vésicules neurales de taille réduite et de forme irrégulière. La formation du rhombencéphale typique n'a été obtenue qu'à partir des greffons et des explantats prélevés au début de la neurulation.

A ces étapes successives d'un développement réalisé par l'ectoblaste soumis à l'action inductrice de plus en plus longue, correspond une progression quantitative nette: plus le contact avec le chordo-mésoblaste a été prolongé, plus grande est la portion du greffon évoluant en crête neurale; ou, si l'action inductrice a été suffisamment longue, plus volumineuses sont les structures neurales induites dans l'ectoblaste greffé.

La seule différence frappante entre les résultats de mes deux séries d'expériences est que: l'ectoblaste soumis à l'action inductrice exercée par le matériel chordal et somitique de même âge que lui n'a jamais fourni des

complexes cérébraux et sensoriels acrencéphaliques; tandis que dans la deuxième série d'expériences, où l'ectoblaste jeune a été mis en contact avec l'inducteur beaucoup plus âgé, de telles structures ont été édifiées par un certain nombre de greffons jeunes et d'âge moyen (laissés sur le substratum inducteur de 16 à 22 heures). Force est donc d'admettre qu'au cours des derniers stades de la gastrulation, le pouvoir inducteur de la région chordale de la voûte archentérique se modifie considérablement. Au début de la neurulation ce matériel embryonnaire acquiert une certaine capacité d'induire dans l'ectoblaste la formation du cerveau antérieur. Cependant la puissance inductrice chordencéphalogène prédomine toujours dans la région deutoméritique de la voûte archentérique. L'induction de l'acrencéphale par ce matériel n'est possible qu'en dissociant artificiellement les effets de l'action inductrice acrencéphalogène de ceux provoqués par le pouvoir inducteur deutencéphalogène, dont le chordo-mésoblaste est toujours doté. Ce dernier résultat peut être obtenu en interrompant le contact inducteur suffisamment tôt et au moment opportun.

SUMMARY

Transplantations were made of pieces of rhombencephalic neurectoderm and presumptive anterior spinal cord to the ventral surfaces of young neurulae of *Triturus alpestris*. These experiments were made in two principal series: in the first, the ectoderm had been exposed to inductor of the same age; in the second, younger gastrula ectoderm had been placed in contact with the anterior chordal region of the archenteron roof of young neurulae (in which the inductor had developed to a stage where, in a normal embryo, the major processes of induction of the nervous system have been completed). In these two experimental series the ectoderm was withdrawn from inductive action more or less precociously, but always before the process of induction had been completed. These transplantation experiments were compared with the development of rhombencephalic explants excised from young neurulae.

The first manifestations of inductive activity (to which the grafts had been submitted before their transplantation) was always the formation of neural crest, dispersed as ectomesenchyme and pigment cells. From older grafts there developed ganglionic masses and, eventually, irregularly formed small-sized neural vesicles. The formation of the typical rhombencephalon has only been obtained from grafts and explants taken at the beginning of neurulation.

There is a clear quantitative progression of the successive stages of development of the ectoderm corresponding to the duration of inductive activity. The more that contact with the chordo-mesoderm was prolonged, the greater the portion of the graft evolving into neural crest, or, if the inductive activity was sufficiently long, the more voluminous the neural structures in the grafted ectoderm.

The sole striking difference between the results of the two series of experiments is that ectoderm subjected to induction by chordal and somitic material of

its own age has never developed acrencephalic complexes of brain and sense organs, while in the second series, in which the young ectoderm was placed in contact with a much older inductor, such structures were formed by a certain number of young and medium aged grafts (left upon the inducing substrate for 16 to 22 hours).

One must thus conclude that during the later stages of gastrulation there is a considerable modification of the inductive power of the chordal region of the archenteron roof. At the beginning of neurulation this embryonic material acquires some capacity to induce the formation of the anterior brain in the ectoderm. It is significant that the chordencephalogenic inductive power is always predominant in the deutomeritic region of the archenteron roof. The induction of the acrencephalon by this material is not possible unless the effects of the acrencephalic inductive activity are dissociated artificially from those provoked by deutencephalogenic inductive activity with which the chordo-mesoderm is always endowed. This last result can be obtained by interrupting the inducing contact sufficiently early and at the right moment.

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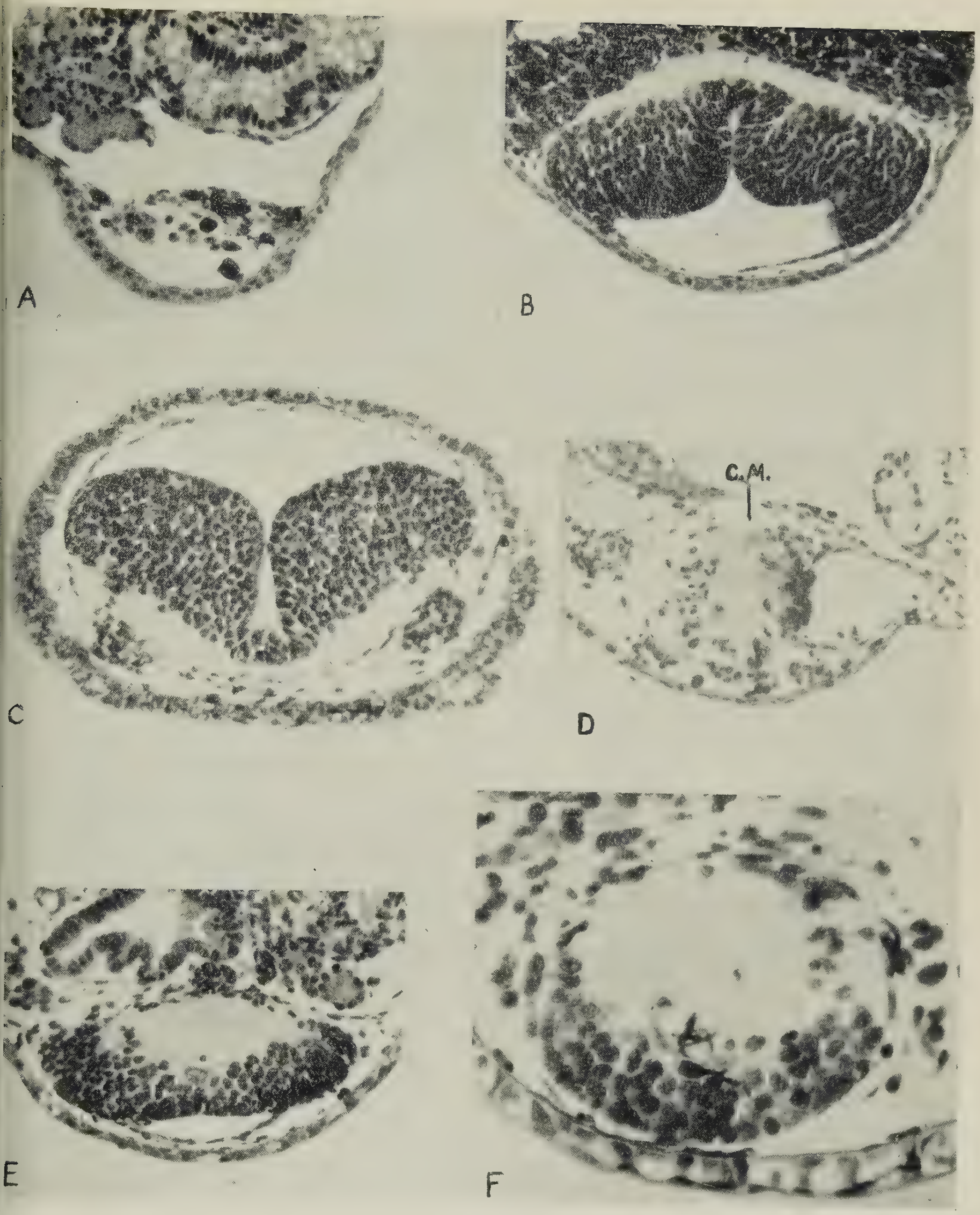
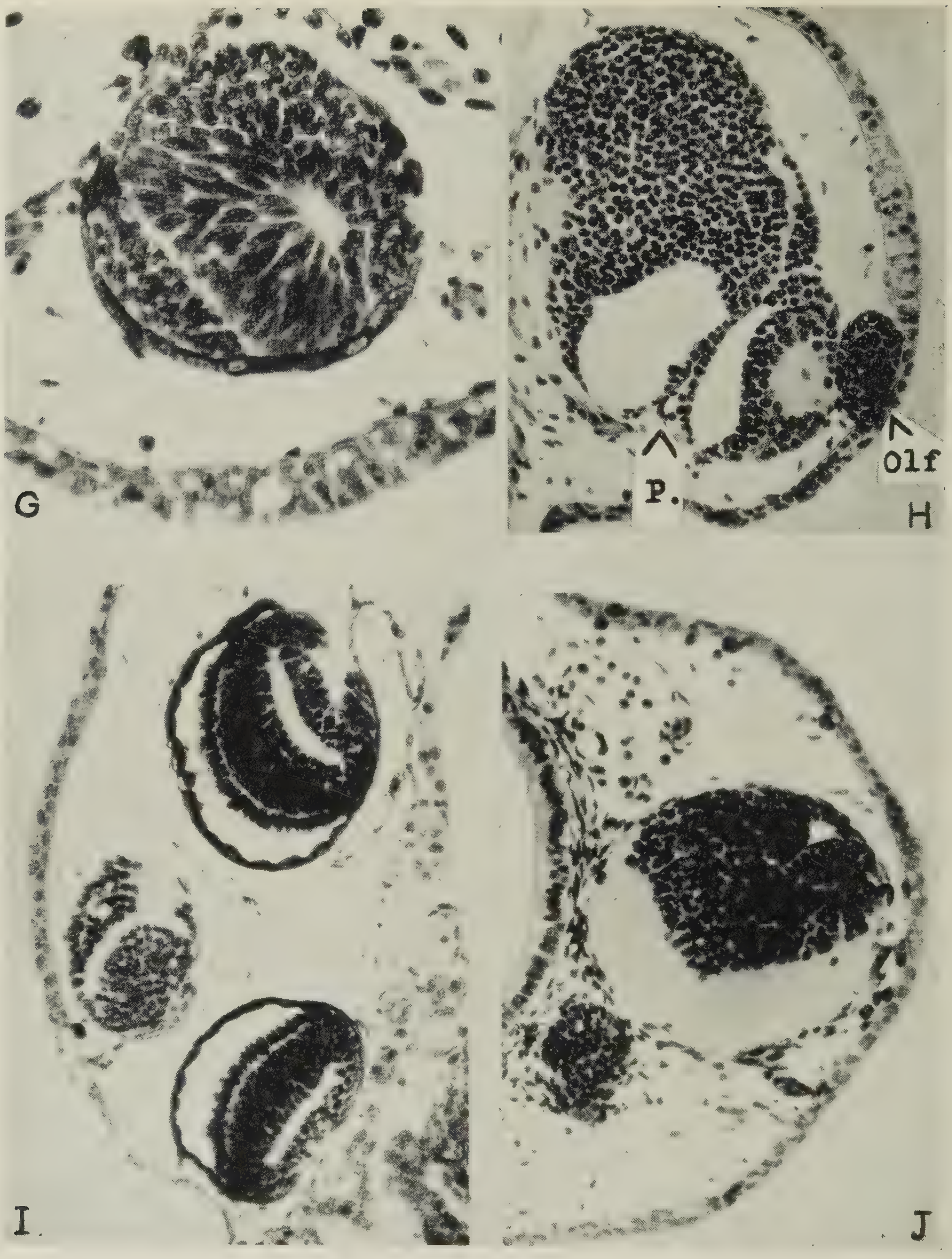


J. GALLERA

Planche 1



J. GALLERA

Planche 2

EXPLICATION DES PLANCHES

PLANCHE 1

FIG. A. Greffon ectoblastique, contenant le matériel rhombencéphalique et médullaire présomptif. Ce greffon, prélevé sur une gastrula munie d'un grand blastopore circulaire, s'est incorporé dans l'épiblaste ventral de l'hôte et a donné de nombreuses cellules ecto-mésenchymateuses et pigmentaires. L'épiblaste formé aux dépens du matériel greffé est plus épais que celui de l'hôte.

FIG. B. Greffon rhombencéphalo-médullaire, prélevé au début de la neurulation. Ce greffon, transplanté sur la face ventrale d'une jeune neurula, a bâti un rhombencéphale relativement bien conformé. La microphotographie reproduit une coupe transversale de ce cerveau.

FIG. C. Coupe transversale d'un explantat excisé de la région chordencéphalique d'une jeune plaque neurale. L'explantat a fourni un beau rhombencéphale accompagné des ganglions et des vésicules auditives (ces dernières ne sont pas visibles sur la coupe reproduite).

FIG. D. Greffon rhombencéphalique présomptif, prélevé sur une gastrula munie d'un petit blastopore circulaire. Le greffon a formé un amas cellulo-fibreux d'aspect neuroïdal, cependant sa nature rhombencéphalique est attestée par la présence d'une cellule de Mauthner (C. M.). Celle-ci est située excentriquement.

FIG. E. Greffon, contenant le matériel rhombencéphalique et, partiellement, médullaire antérieur, excisé d'une neurula au stade de l'apparition du liséré pigmenté signalant la limite de la plaque neurale. Le greffon a formé un rhombencéphale de taille réduite et de forme caractéristique pour les monstres otocéphales. Une seule cellule de Mauthner s'est constituée; elle occupe une position strictement médiane dans le plancher du rhombencéphale.

FIG. F. Fragment de l'ectoblaste jeune laissé sur la voûte archentérique de l'hôte intermédiaire pendant 24 heures et 30 minutes. Ce greffon, repris et transplanté sur l'hôté définitif, n'a formé qu'un cordon cellulo-fibreux entouré de nombreuses cellules ecto-mésenchymateuses et pigmentaires. Quelques-unes de ces dernières cellules ont pénétré à l'intérieur du cordon neural, la barrière habituelle constituée par la limitante gliale externe étant absente.

PLANCHE 2

FIG. G. Le greffon a été laissé pendant 18 heures sur le chordo-mésoblaste de l'hôte intermédiaire. Après la transplantation définitive, il a donné un œil, petit mais parfaitement constitué, et un nodule neural (invisible sur la coupe reproduite). Ce dernier est relié à l'œil par un cordon fibreux.

FIG. H. Un lambeau d'ectoblaste jeune a été reporté sur la voûte archentérique d'une jeune neurula. Après 18 heures et 30 minutes le greffon a été retransplanté sur la face ventrale d'une autre neurula. Ce greffon a bâti un cerveau antérieur entier, accompagné d'une ébauche oculaire rudimentaire. La coupe reproduite est très oblique par rapport au cerveau. On voit le télencéphale, une petite placode olfactive (Olf.) et, enfin, une paraphyse (P.).

FIG. I. Le greffon ectoblastique jeune, après avoir été soumis durant 21 heures et 45 minutes à l'action inductrice du chordo-mésoblaste plus âgé, a été transplanté sur la face ventrale d'une jeune neurula. Il s'est développé en cerveau entier, quoique de forme irrégulière. Sur la coupe reproduite on voit les deux yeux et l'extrémité antérieure du cerveau.

FIG. J. Le greffon ectoblastique, soumis à l'action inductrice du chordo-mésoblaste âgé durant 21 heures et 30 minutes, a édifié un court tronçon de moelle (allongée ou rachidienne) et a fourni de nombreuses cellules ecto-mésenchymateuses, pigmentaires et ganglionnaires. Ces dernières se sont assemblées en formant un puissant ganglion. La moelle est du type 'basal' (Holtfreter, Takaya).

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Enhancement of Growth of Chick Host Spleens following Chorio-allantoic Membrane Grafts of Homologous Tissues[1]

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WITH ONE PLATE

INTRODUCTION

THE phenomenon of enlargement of the host chick embryo spleen, following grafts of homologous adult chicken tissues to the chorio-allantoic membrane (CAM), affords the investigator an excellent tool for the study of growth.

Initial observations of this phenomenon were made by Danchakoff (1916) and Murphy (1916). Grafts of adult chicken spleen on the chorio-allantoic membrane of 9-day-old chick embryos brought about a striking enlargement of the host spleens after 8 additional days of incubation. The phenomenon was later studied by Ebert (1951), who showed that the effect was only partially organ-specific. Grafts of thymus and liver affected the weight of the host spleen, but in each case the effect was far smaller than that observed with splenic transplants. Andres (1955) found that injected kidney and liver debris, which elicited an increased mitotic index in the homologous host organ, was not inhibited in its action by killing the cells through freezing and subsequent thawing. Van Haeften (1958) recently reported hypertrophy of the embryonic host spleen following the administration of cell-free homogenates of spleen to the CAM.

The above findings may be interpreted as being in accord with earlier template-antitemplate hypotheses (Weiss, 1947; Tyler, 1947): the introduced template, supplementing existing cellular templates, catalyse the formation of additional antitemplates leading to the enlargement of the homologous organ. Intact and proliferating cells as such, were sufficient but not necessary to produce the enlargement.

Ebert (1954), using adult chicken spleen and kidney tissues labelled with S^{35},

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[1] Scientific Paper No. 1821, Washington Agricultural Experiment Station, Pullman; Project Nos. 717 and 1255. Presented in part at the annual meeting of the American Society of Zoologists, 24–28 August 1958, Bloomington, Indiana.

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observed a selective incorporation of radioactivity into the proteins of homologous tissues of the host chick embryos. He envisaged a transfer of 'tissue specific protein constituents larger than amino-acids' from the grafts into proteins of the host tissue, and thus strongly suggested a 'building block' mechanism rather than a template or catalytic mechanism.

More recently, Ebert (1957, 1958 *a*, *b*, 1959) demonstrated that radioactively labelled (S^{35} methionine) nonviable homogenates of organs injected into the vascular system of the 9- or 10-day chick embryo were differentially incorporated in the homologous organ. Examination of nuclear, mitochondrial, microsomal, and supernatant fractions of the labelled homogenates showed clearly a localization of only the microsomal and supernatant fractions, and not of the nuclear and mitochondrial fractions. Ebert emphasized, however, that these experiments 'deal only with one aspect of the problem, namely predominant localization'.

In 1951 Ebert reported that the ability to evoke enlargement of the host embryo spleen was correlated with an increase in the number of antigens in the developing donor spleen. Grafts from embryonic chick donors of less than 14 days' incubation did not elicit enlargement of the homologous organ of the host. However, grafts from older embryonic donors produced a significant increase in the weight of the host spleen.

Recently, Simonsen (1957) presented evidence which suggested that intact donor cells capable of proliferation and antibody production were necessary in this phenomenon of host spleen enlargement. He observed an enlargement of the host spleen following injections of suspensions of chicken spleen cells or whole adult chicken blood into chick embryos on the 18th day of incubation. When similar experiments were performed on inbred lines of mice, the host spleen was not enlarged following the injection of cells from donors of the same strain. This observation was also made later in the chicken (Cock & Simonsen, 1958).

Cock & Simonsen (1958) postulated that the injected donor cells proliferate, colonize the reticulo-endothelial system of the host, and, responding to the antigenic stimulus presented them by the host, proceed to attack the host cells 'both by producing antibodies against the host erythrocytes, and by means of a homograft reaction'. Simonsen (1957) had previously found, 'by means of Coombs' test', antibodies against blood group antigens of the recipient in the blood of the recipient.

DeLanney and Ebert (see Ebert, 1958 *a*, *b*, and DeLanney, 1958) have also presented striking evidence of the graft-*versus*-host reaction, namely, destruction of the homologous organ of the host and subsequently the host embryo itself, following grafts of immunologically competent adult tissues. Whether the grafts were made to the chorio-allantoic membrane or to the coelom of the chick, or to the coelom or dorsal fin of the salamander, the basic results have been consistent with the hypothesis of a graft-*versus*-host reaction. Ebert (1958 *a*, *b*) has made it clear that although the occurrence of the graft-*versus*-host reaction does not necessarily argue against the hypothesis of organ-specific growth regulation, it does make tests of the latter hypothesis difficult when immunologically competent tissues are employed.

The original objective of the present investigation was to determine whether the antigen-antibody mechanism was involved in this enlargement of the host embryo spleen following CAM transplant of adult chicken spleen tissue. The cells of the donor were inactivated, i.e. rendered incapable of mitosis, by freezing and subsequent thawing, and by X-irradiation of the tissues prior to implantation on the CAM. In a second series of experiments, the activity of the donor spleen tissue when implanted on hosts from the same inbred line, was compared with its activity on hosts from a different inbred line of chickens. In a third series of experiments an attempt was made to increase the activity of the adult donor spleen tissue (1) by pre-injection with embryo spleen tissues, and (2) by the implantation of spleens from more distantly related animals, e.g. the turkey and the pheasant.

METHODS AND MATERIALS

'Synthetic' dominant white and White Leghorn chickens and eggs were used in this investigation. Eggs were incubated for 9 days at 99·5° F.

The method of transplantation of tissues to the chorio-allantoic membrane (CAM) adopted in this study was essentially that described by Hamburger (1942). A quadrilateral window (1 × 1 cm.) was cut in the shell with a hack-saw blade. The shell membrane was punctured and reflected, and a piece of tissue approximately 1 × 1 × 2 mm. (weight 5 to 10 mg.) was placed on the CAM. The shell membrane and the piece of cut shell were replaced and covered with a plastic tape. The edges of the tape were sealed with paraffin wax. The eggs were placed in the incubator with the small end down.

The operated eggs were incubated for 8 additional days. On the 17th day of incubation the grafts were examined and each graft was graded, independently of the size of the host spleen, according to its condition as follows:

1. *Fully successful*: graft well vascularized, pinkish, and as large as, or larger than, the original piece of grafted tissue.
2. *Partially successful*: graft pink but smaller than the original graft.
3. *Not successful*: graft brown or green, apparently without blood-vessels coursing through it. The darkened and somewhat shrunken piece of tissue can be readily dislodged from the CAM.

The spleens of the embryos were then removed and washed in saline. After excess moisture was removed, the spleens were weighed to the nearest 0·2 mg. on a Roller–Smith torsion balance.

The findings reported in these studies are based on 33 experiments involving more than 3,000 embryos.

Each experiment included unoperated controls and embryos in which 0·05 to 0·1 c.c. of chick Ringer's solution was dropped on the chorio-allantoic membrane as well as embryos which received grafts. Because adult chicken kidney tissue did not significantly affect the size of the host spleen, such grafts frequently also served as controls. The eggs were coded and subjected to the various treatments in a random sequence.

RESULTS

Experiment 1: The effects of frozen spleen grafts on the homologous host organ

Fragments of fresh adult chicken spleen, liver, and kidney tissue were frozen in 15 to 30 seconds by placing them on the holder of a CO_2 freezing microtome and permitting the gas to escape rapidly. Tissues were also frozen by placing them in a freezer (—20° C.) for 1 to 5 days. Prior to implantation, the tissues were thawed at room temperature, and were either cut into small pieces (1 × 1 × 2 mm.) or macerated with an equal amount of saline in a conical test tube with a fitted and ground glass rod. Microscopical examination of the macerated tissue showed intact cells, both singly and in clumps. In some cases, fresh adult chicken spleen was heated in a water bath at 56° C. for an hour before implantation.

TABLE 1

Effect of frozen and heated adult chicken tissue on the weight of the chick embryo spleen

	Fully successful		*Partially successful*		*Not successful*	
Donor tissue	*Percentage*	*Av. wt. of host spleen*	*Percentage*	*Av. wt. of host spleen*	*Percentage*	*Av. wt. of host spleen*
		(mg.)		(mg.)		(mg.)
Fresh spleen . . .	79 (129)*	26·8	13 (22)	18·2	8 (13)	15·4
Frozen spleen . . .	13 (18)†	10·7	38 (53)	10·4	49 (69)	9·7
Heated spleen . . .	0 (0)	. .	13 (1)	8·0	87 (7)	10·5
Fresh liver . . .	20 (2)	42·3	40 (4)	20·4	40 (4)	16·6
Frozen liver . . .	32 (8)	8·7	44 (11)	8·2	24 (6)	9·1
Fresh kidney . . .	57 (33)	14·3	21 (12)	11·9	22 (13)	11·3
Frozen kidney . . .	9 (3)	15·3	48 (16)	11·1	42 (14)	10·8
Not operated controls .	(81)	9·2	. .	. .	. .	. .
Saline controls . . .	(71)	11·7	. .		. .	. .

* Figures in parentheses indicate No. cases.

† Refer to experiment 1 in text.

Macerated or crushed fresh (unfrozen and unheated) spleen (5 to 10 mg. of tissue) gave a higher percentage of successful incorporation on the CAM than frozen or heated spleen—as the presence, usually, of 2 or 3 large nodules on the CAM demonstrated. In these cases the host spleens also tended to be more uniform in size and appearance. The frozen and heated tissues, both whole and macerated, when separated from the CAM were soft and crumbly, and white or yellowish in colour. The host spleen size was not significantly enhanced, even in those cases in which the grafts may have been considered 'successful' (Table 1). These 'successful' grafts appeared to be well vascularized and were as large as the original pieces of implanted tissue. However, sections of these grafts showed large areas of necrosis and no detectable mitotic figures.

These findings are supported fully by a similar analysis of over 400 frozen and alcohol-fixed spleen grafts by Ebert (personal communication).

However, in view of the low percentage of fully successful 'takes', one cannot yet conclude that the frozen and heated donor spleen tissue cannot influence the size of the host spleens. The inability to stimulate the host organ may have been due simply to the failure to 'take'.

Although fresh chicken liver tissue stimulated growth of the host spleen, frozen liver tissue did not. Fresh and frozen kidney tissue did not stimulate the host spleen to a significant extent.

Experiment 2: The effects of X-irradiated spleen grafts on the homologous host organ

Because a majority of the frozen spleen tissues failed to 'take' successfully on the CAM, another method was sought which would destroy the ability of the cells to proliferate without reducing the ability of the tissues to 'take' successfully on the CAM.

X-irradiation of the adult spleen tissue before implantation was tried because it is well recognized that at high doses it can block or inhibit mitosis. Such an approach should, therefore, be useful in demonstrating whether donor cells capable of undergoing mitosis are necessary for host spleen enlargement.

Accordingly, fragments of adult chicken spleen and kidney tissues, approximately 10 × 5 × 2 mm., were irradiated for 5 to 60 minutes with a therapeutic-type X-ray machine, operating at 250 kV and 15 mA. A 0·5 mm. Cu filter was used, giving a filter factor (HVL) of 1·38 mm. Cu. The tissues were irradiated at a rate of 41 r/min., as measured with a Victoreen r meter, and at a distance of 50 cm. from the source. The tissues were kept below 10° C. in a water bath during handling and X-irradiation to reduce autolysis during the long period of exposure.

After irradiation, the tissues were cut into smaller pieces and implanted on the CAM. A specimen of each irradiated tissue was fixed in Baker's calcium formol or Bouin's fixative for further histological study.

The effect of X-irradiation on the ability of the tissues to stimulate growth of the host spleen is summarized in Table 2.

These results indicate that:

1. At lower doses (between 205 and 820 r) the X-irradiated adult chicken spleen stimulated the growth of the host chick spleen. Microscopical examination of sections of well incorporated grafts showed many normal appearing spleen cells. Mitotic figures ranged between 3 and 5 per cent. (Plate, figs. 1, 2).
2. At higher doses (between 1,230 r and 1,845 r), a significant decrease was observed in the ability of the donor tissue to influence the size of the host spleen. Microscopical examination of sections of successfully incorporated grafts

showed a few well-stained and apparently normal spleen cells. Mitotic figures, however, were relatively rare, i.e. between 0 and 1 per cent. (Plate, figs. 3, 4).

3. At doses above 1,845 r, no stimulation of the host spleen was observed. Many of the X-irradiated grafts were embedded in, or enclosed by, the chorio-allantoic membrane. The majority of the grafts were necrotic. Microscopical examination of sections of the irradiated grafts showed many dark, small, pycnotic nuclei (Plate, figs. 5, 6). The percentage of successful grafts was decreased.

TABLE 2

Effect of X-irradiated chicken spleen and kidney tissue on the homologous host chick organ

		Fully successful cases only		
Donor	*Dose*	*Percentage*	*Number of cases*	*Average weight of host spleen*
	(r)			(mg.)
Spleen	0	70	48	37·5
	205	67	16	32·5
	410	80	37	25·2
	820	75	27	25·2
	1,230	65	22	18·1
	1,845	46	5	10·3
	2,050	50	5	11·6
	2,460	15	6	10·4
	0 and 2,460*	69	11	28·54
Kidney	0	59	28	15·0
	1,845	47	22	10·7
Saline	0	..	84	9·6

* Two grafts transplanted concomitantly to the same host.

This experiment shows that adult chicken spleens, subjected to high doses of X-irradiation (1,230 r to 1,845 r) prior to transplanting to the CAM, lose the capacity to increase the weight of host spleens, even though they are well incorporated as grafts and appear to be normal. Grafts of 'heavily' irradiated (2,460 r) spleen and kidney tissue did not result in spleen weights which were less than that of normal or saline controls. Moreover, concomitant implantation of both non-irradiated and irradiated (2,460 r) spleen brought about the characteristic increase in the size of host spleens.

The ability to stimulate growth of the host spleen is thus apparently correlated with the number of mitotic figures observed in sections of the X-irradiated graft. It is well recognized that cellular proliferation is important in antibody formation (Fagraeus, 1948; Harris, Harris, Beale, & Smith, 1954), and that X-irradiation can depress or inhibit antibody formation (Taliaferro & Taliaferro, 1951; Hale & Stoner, 1954). These observations suggest, therefore, that the antibody-forming mechanism may be involved in host spleen enlargement. However, a direct test for the ability of the X-irradiated tissue to produce antibody was not conducted.

Experiment 3: Comparison of growth stimulating effects of spleens from two inbred lines of chickens

The previous experiment demonstrates that X-irradiation of adult chicken spleen tissue can destroy its ability to stimulate growth of embryonic spleen. Many workers (e.g. Hale & Stoner, 1954) have reported that X-irradiation can destroy antibody formation. Accordingly, a study was made of the possible role of immunological mechanisms in this reaction.

Adult spleens from two inbred lines of White Leghorn chickens, selected for leukosis susceptibility (*S*) and leukosis resistance (*R*) were implanted, reciprocally and within lines, on the CAM of embryos from these two lines. If immunological mechanisms are involved, one would expect a greater reaction between the two lines than within each line. Although the two lines were not exactly of the same degree of inbreeding, both have been maintained, with a limited number of matings and without introduction of outside stock, for over 20 years. The experiments were repeated three times, with essentially similar results.

TABLE 3

Effect of donor spleens from two inbred lines of chickens on the spleen of the host chick embryo

Adult donor	*Egg host*	*Fully successful cases only* *Number of cases*	*Average weight of host spleen*	*S.E. of mean*
			(mg.)	
*S**	S	18	26·4	3·346
R†	S	23	18·6	1·991
Saline	S	27	10·1	0·381
S	R	46	45·5	3·648
R	R	28	13·45	1·069
Saline	R	52	9·88	0·333
S	S×R	13	47·4	5·85
R	S×R	9	16·0	1·96
Saline	S×R	12	9·4	2·94

* *S* line selected for leukosis susceptibility.
† *R* line selected for leukosis resistance.

Spleens from adults of the *S* line brought about a lesser stimulation of the spleens in embryos from the *S* line than in embryos from the *R* line. Similarly, spleens from the *R* lines brought about a lesser stimulation in *R* embryos than in *S* embryos. On the basis of a pooled *t* test, the differences were significant at the 1 per cent. and 5 per cent. levels respectively (Table 3).

When spleens from adults of the *S* line were implanted on the CAM of the F_1 ($R \times S$) embryos, an enlargement of the host spleen was observed which was similar in magnitude to that obtained following grafts of *S* line spleens on *R* line embryos. Spleens from *R* line adults, when implanted on the CAM of the F_1

($R \times S$) embryos, did not produce as great an enlargement as those on *S* line embryos. This experiment was not repeated, due to the unavailability of material.

It was further noted that spleens from adult *S* line chickens consistently brought about a correspondingly greater increase in the spleen of the host (regardless of the latter's origin) than spleens from adult *R* line chickens. Similar effects were noted in this laboratory in 1957 (unpublished) when the growth-stimulating effect of spleens from the same *S* line was compared with that produced by spleens from a non-inbred flock of White Leghorn chickens.

The two lines used were not checked for 'effective homozygosity' by skin grafting. However, the two lines are apparently sufficiently different to respond characteristically and consistently to the different treatments.

These results are essentially in agreement with those of Cock & Simonsen (1958), who used two highly inbred White Leghorn lines. In their study, gross enlargement of the spleen and liver resulted when blood from adult birds of an inbred line (*I*) was injected into newly hatched chicks of a cross between two highly inbred lines (*C* and *I*). On the other hand, F_1 blood injected into F_1 chicks, and either *I* or F_1 blood injected into *I* chicks, produced only a relatively slight enlargement of the spleen and liver.

Cock & Simonsen concluded that the observed enlargement was due to the multiplication of donor cells within the host and the launching by them of an immunological attack against the host. Simonsen (1957) had previously demonstrated the presence of antibodies against blood group antigens of the host in the blood of the host.

Results of the present study (experiment 3) suggest that an antigen-antibody type mechanism may be involved. This led to the question: could this reaction be increased (1) by exposing the adult chicken donor to the chick embryo spleen (thus stimulating antibody production) prior to implantation on the CAM or (2) by the grafting of spleens from such animals as the pheasant, turkey, rat, or guinea-pig (which would be expected to differ antigenically from the host chicken embryo)?

Experiment 4: The effect of spleens from adult chickens injected with chick embryo spleen prior to implantation on the CAM

To test the first possibility mentioned above seven adult White Leghorn and 'synthetic' dominant white hens were injected with homogenized frozen and fresh spleens from embryos incubated for 15–19 days. At least 10 injections, over a period of not more than two months, were given to each animal. Approximately 10 to 15 mg. of tissue were injected into each animal intravenously or intra-peritoneally. The animals were bled and killed one week after the last injection.

As shown in Table 4, spleens from chickens injected with chick embryo spleens did not produce a greater increase in the size of the host embryo spleen.

Instead, the stimulating effect of spleen from the injected chickens was consistently and significantly less (at the 1 per cent. level, using pooled *t* test) than that of spleens from non-injected chickens.

TABLE 4

Effect of spleen from donors injected with 15- to 19-day chick embryos

Donor	*Fully successful cases only*		
	Number of cases	*Average weight of host spleen*	*S.E. of mean*
		(mg.)	
Non-injected .	78	33·08	3·034
Injected . .	97	24·21	1·288
Saline . .	56	9·53	0·247

If circulating antibodies to the injected antigens are involved in the stimulation, one would have expected a greater response from the injected donor spleens than that obtained from the non-injected donors. This was not the case.

However, the titres of the antisera, obtained in this laboratory by interfacial 'ring' tests, were low or negligible (1:4). The low titres of these antisera may have been due to the weak antigenicity of the chick embryo spleen, as well as to the antigenic composition of the chick spleen, which may be similar to that of the adult chicken. Ebert (1951) reported that by the 18th day of incubation all antigenic components present in the adult can be found in the embryo.

This problem is being further investigated in this laboratory by using antisera prepared in one line of White Leghorn chickens against the spleen from a second inbred line, combined with complement according to a procedure described earlier (Mun, 1958). It is possible that the injected homogenates of the embryos fail to supply antigens to the adult chicken in the same form in which they are made available to the donor graft by the host embryo.

Experiment 5: Effect of spleens from different animal species on the growth of the chick embryo spleen

To test the possibility that the hypertrophy of host spleens might be significantly increased by the use of *heterologous* donor material, spleens from pheasants, turkeys, guinea-pigs, and rats were implanted on the 9-day-old chick embryo CAM as described above.

The turkey and pheasant spleen grafts were incorporated in the chorio-allantoic membrane. In some instances the grafts were more than 2 to 3 times the size of the original piece of implanted tissue. The turkey and pheasant spleen tissues affected the size of the chick host spleen to a significant degree (0·1 per cent. and 1 per cent. levels, respectively). However, these effects were not greater but significantly less (at the 5 per cent. and 1 per cent. levels, respectively) than that of adult chicken spleen grafts on the homologous host organ (Table 5).

Both rat and guinea-pig spleen grafts, on the other hand, did not 'take' well and, probably for this reason, did not influence the size of the host spleen. The reason for this failure to 'take' is not known.

TABLE 5

Effect of spleen from different animals on the weight of the chick embryo spleen

	Fully successful cases only			
	Percentage	*Number of cases*	*Average weight of host spleen*	*S.E. of mean*
			(mg.)	
Chicken . . .	67	41	26·0	2·434
Turkey . . .	79	19	16·6	1·525
Pheasant. . .	86	17	12·8	1·582
Rat . . .	6	8	12·2	. .
Guinea-pig . .	5	8	11·2	. .
Saline . . .	. .	46	9·14	0·328

The existence of a natural antibody in the rejection of mouse tumour-cells by the chick embryo has been reported by Green & Lorincz (1957). They showed that the death of the injected tumour-cells and the appearance in the chick embryo of serum gamma globulins occurred on the 17th day of incubation. In our study, however, the tissues were implanted on the 9th day of incubation and removed on the 17th day, and it is therefore unlikely that the failure of the rat and guinea-pig spleen graft to 'take' was due to a natural immunity.

Thus, although the grafts of turkey and pheasant spleen were well incorporated on the CAM, the host chick spleen was only slightly enlarged. As has already been stated, in view of the greater genetic diversity between the turkey and the chick host, a greater stimulation of growth might have been expected if the host spleen enlargement is due to an antigen-antibody type reaction.

DISCUSSION

The available data suggest that the phenomenon of growth stimulation of the host chick embryo spleen by homologous adult organ tissues involves at least two steps: (1) the initial stimulation leading to increased mitotic index and a subsequent enlargement of the host organ, (2) death of the host following debilitation and subsequent atrophy of the spleen and hematopoietic organs. The first step has been studied extensively by the students of growth; the second was unexpectedly encountered by workers in embryology and radiation protection. Although there may be common basic mechanisms underlying the two, each step may have its own peculiar mechanism.

A diffusible substance, resistant to the effects of freezing and subsequent thawing, may be involved in the first step. This was clearly demonstrated in the experiments of Andres (1955) where an increased mitotic index in the liver and kidney was observed following injection of frozen and thawed non-viable homologous organ debris. The work of Ebert (1958 *a*, *b*) suggests that the growth stimulating substance(s) may be identified with the microsomal and supernatant fractions of radioactively labelled (S^{35} methionine) non-viable homogenates of organs. Continuous stimulation by substances of this sort may eventually bring about an increase in the size of the spleen, although this has not been actually demonstrated.

The failure of the frozen spleen material to stimulate growth of the host spleen (experiment 1) does not contradict this possibility of a diffusible growth stimulating factor or factors. The failure to stimulate may be due to the failure to 'take' and thereby supply the host spleen with a continuous flow of stimulating substances. Van Haeften (1958), using larger amounts of cell-free spleen homogenates (200 to 500 mg. of spleen homogenate) was able to observe an enlargement of the host spleen. If corroborated by subsequent work, his report is of profound interest, but it must be noted that the criteria for the absence of intact cells in the homogenates are not altogether clear; the use of filters is not reported.

In view of this report that spleen enlargement can be brought about in the absence of intact donor cells, it is difficult to picture the resulting enlargement as being exclusively due to a massive migration of intact cells from the donor to the host.

In a study employing radioactive tracers, Ebert (1954) presented three lines of evidence militating against this possibility:

(1) Microscopic examination of histologic preparations of embryonic host kidney failed to reveal any accumulation of cells which was not incorporated into the normal cellular pattern of the kidney.

(2) A study of autoradiograms of grafts of kidney, spleen, and other tissues of host embryos indicated that while there was, in general, a higher level of radio-sulphur in the homologous tissues, there was no localization in any region or in any specific cell type of the kidney or spleen.

(3) Analysis of DNA in enlarged and normal spleens showed that the DNA content of the enlarged spleens did not increase concomitantly with the increase in protein content.

The hypotheses of Weiss (1947) and Tyler (1947) have been frequently invoked in the interpretations of these events. However, the possibility of differentiation and rapid proliferation of a 'seed' population of donor cells in the specialized host environment cannot be discounted.

It appears, at first glance, that the observations of Cock & Simonsen (1958) on highly inbred lines, and the results in experiment 3 of this study, cannot be adequately explained in terms of a template-antitemplate mechanism. However, it must be noted that even in the use of highly inbred material, such as was available to Cock & Simonsen (1958), there was a *slight enlargement* of the host spleen when blood from pure *I* adult fowls was injected into *I* chicks. In experiment 3 of our studies, when *S* and *R* adult spleens were grafted on embryos from the same line, there was also a slight but significant increase in size of the spleen.

Thus, the hypotheses of Weiss and Tyler cannot be completely discounted. This slight increase, in our case, may be due to antigenic differences between the two lines.

The severe anaemia, local reactions at the site of injection, and death of the host chick observed by Cock & Simonsen (1958) can very well be explained by a graft-*versus*-host-type of reaction. This step, however, is probably secondary and may be considered as separate from the first 'stimulation' step described above. The second step requires intact cells (Simonsen, 1957).

In our studies a small proportion of the embryos receiving CAM grafts of adult chicken spleen tissue were apparently afflicted with an immune reaction. Approximately 10 per cent. of the enlarged spleens appeared mottled and showed areas of necrosis in section. A similar proportion of the chicks receiving grafts of adult chicken spleen on the 9th day of incubation were small or stunted (approximately 50 per cent. of body-weight of the controls at 4 to 6 weeks' post-hatching). However, the bulk of the enlarged spleens and hatched chicks appeared normal. The role of haemorrhage, infection, and rate of healing associated with the CAM grafting technique remains to be examined.

In our attempt to discover how the grafted donor spleen tissue affects the host spleen we observed that the reaction is not aggravated by methods which are supposed to increase the amount of circulating antibodies against the host spleen or to incite a greater immunologic reaction. On the contrary, the response is in the opposite direction. Thus, although it appears that the host must be challenged by immunologically competent tissues, the mechanism involved in the subsequent enlargement is probably not dependent on antibody activity. This interpretation is, of course, based primarily on evidence obtained by the use of the CAM transplant technique. Indeed, the failure to obtain the expected response in experiment 5 may be due to the inability of the donor cells to colonize the host spleen in sufficient numbers. Experiments are under way in this laboratory to test this possibility, viz., the injection of homogenate of turkey spleen into the chick embryo.

SUMMARY

1. A striking enlargement of the host chick embryo spleen was obtained following chorio-allantoic grafts of adult chicken spleen tissue.

2. When the adult chicken spleen tissue was killed by freezing and thawing before implantation on the chorio-allantoic membrane, no enlargement of the host chick embryo spleen was observed. However, a large number of the frozen grafts failed to 'take'.

3. X-irradiation of the donor spleen, at a level sufficient to block or inhibit mitosis, also removed the growth-stimulating abilty of the grafted tissue in spite of the fact that the grafts were well incorporated. This suggested that donor cells capable of undergoing mitosis were necessary for producing enlarged host spleens.

4. Because of the possibility that antibody-forming mechanisms were destroyed at these levels of irradiation, the involvement of such mechanisms in this reaction was studied.

5. In a comparison of the effect of spleen grafts between two inbred lines of White Leghorn chickens (*S* and *R*), a lesser stimulation was obtained when spleens from *S* line chickens were implanted on *S* line embryos than on *R* line embryos. Similarly, a lesser stimulation was obtained when *R* spleens were implanted on *R* embryos than on *S* embryos. Spleens from *S* line chickens also evoked a greater response than spleens from *R* line chickens.

6. To test the role of antibodies in this reaction, spleens from adult chickens previously injected with chick embryo spleens were implanted on the chorio-allantoic membrane. In all cases (7) the spleens from the injected chickens stimulated the host embryo spleen to a significantly lesser degree than spleens from non-injected chickens.

7. Spleens from different animal species, including the turkey, pheasant, rat, and guinea-pig, were implanted on the chorio-allantoic membrane of the early chick embryo. The turkey and pheasant spleens stimulated the host chick embryo spleens to a significantly lesser degree than adult chicken spleen in spite of the greater genetic diversity and subsequently successful incorporation in the chorio-allantoic membrane.

8. From the available evidence the authors suggest that the enlargement of the host chick embryo spleen following chorio-allantoic membrane grafts of homologous adult chicken tissue probably involves two separate steps: (1) a stimulation which may occur in the absence of intact cells; (2) an immune-type reaction which results in necrosis of areas in the host spleen, and stunting of growth of the hatched chicks.

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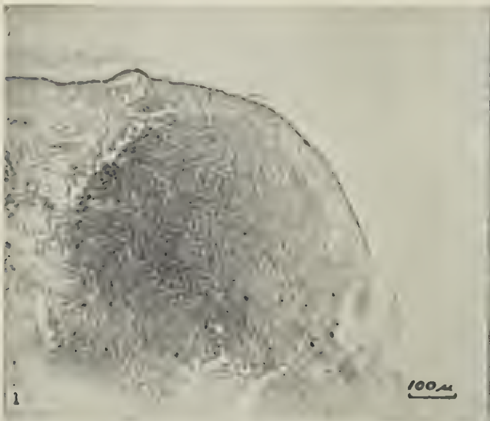

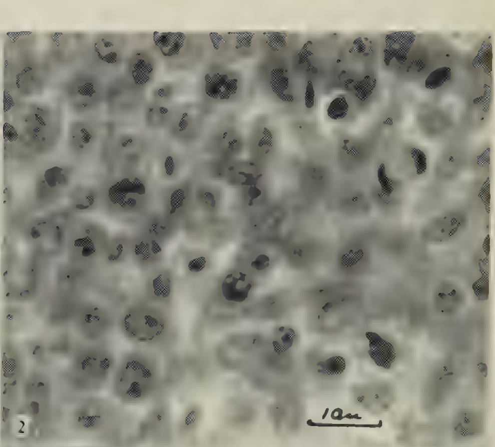

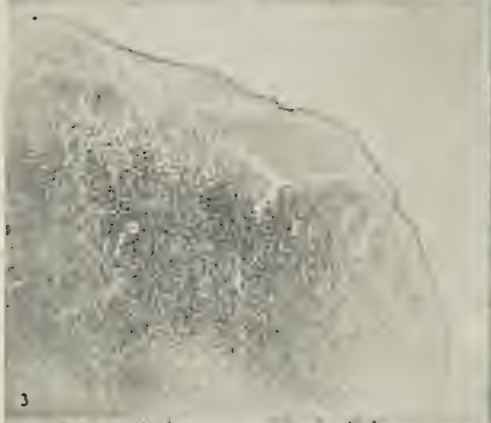

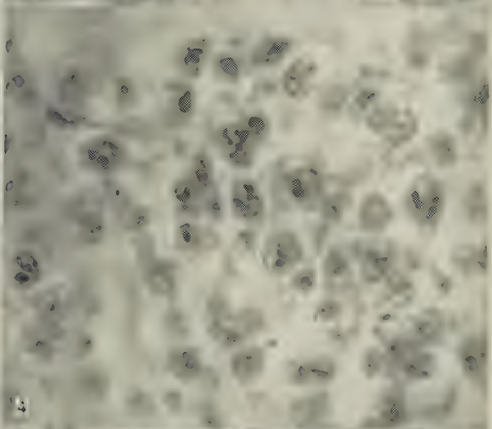

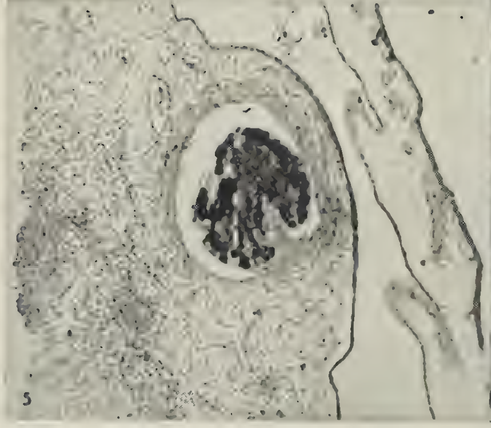

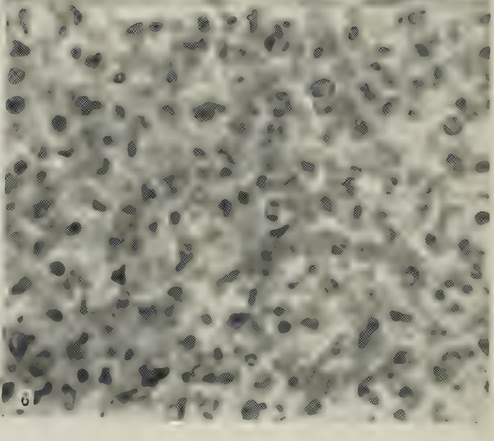

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EXPLANATION OF PLATE

Fig. 1. Section of well-incorporated graft of non-irradiated adult chicken spleen. ×60.

Fig. 2. Section of above graft at higher magnification showing mitotic figures. ×950.

Fig. 3. Section of well-incorporated graft of adult chicken spleen irradiated for 30 minutes (1,230 r) prior to implantation. ×60.

Fig. 4. Section of above graft at higher magnification. ×950.

Fig. 5. Section of well-incorporated graft of adult chicken spleen irradiated for 1 hour (2,460 r) prior to implantation. ×60.

Fig. 6. Section of above graft at higher magnification. ×950.

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Action of Triethanomelamine (TEM) on Early and Later Stages of Mouse Embryos

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WITH FOUR PLATES

SEVERAL years ago triethanomelamine (2,4,6-tri(ethyleneimino)-1,3,5-triazine, or TEM) was introduced into clinical chemotherapy for its tumour-inhibiting property. It was found, in addition, that TEM is a strong mutagenic factor.

In a previous paper (Jurand, 1958) it was shown that TEM severely retards the development of the chick in the early embryonic stages. This effect is directly proportional to the dose of TEM used. It was suggested that the sensitivity of individual embryonic tissues varies in the early stages of development, somites being the most sensitive embryonic organs. There was observed also, especially with larger doses of TEM, a considerable widening of the embryonic coelom and decomposition of the mid-trunk somites into mesenchyme-like loose tissue. There was, moreover, a considerable increase in cell size, and both the nuclei and the nucleoli in the nervous tissue were enlarged.

In the experiments to be described the influence of TEM on mammalian (mouse) embryonic development, and in particular its lethal effect on developing embryos and the changes it causes in embryonic tissues and organs, is investigated.

MATERIAL AND METHODS

The inbred strain JU and the outbred strain JC of the laboratory mouse were used. Strain JU, homozygous for *aa* and *cc*, was recently described by Falconer (1958). Strain JC has been observed at this Institute for a few years and is characterized by different non-homozygous colour genes present in its ancestry (*si*, *m*, *a*, *b*, *d*, *ln*, *bt*, c^{ch}, c^{e}, and *c*). It also shows the non-homozygous gene *Ca* and one skeletal gene, short-ear (*se*). Both of these strains are believed to be free from any mutant genes causing abnormalities which could interfere with the present experiments.

Females, 2–5 months old, were kept with males overnight, and on the next morning were examined for the presence of copulation plugs in the vagina. The day on which the plugs were found was considered to be the first day of preg-

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nancy. For convenience, stages of treatment and age are referred to as 5th, 6th, 9th, &c., but strictly speaking these stages should be regarded as $4\frac{1}{2}$, $5\frac{1}{2}$, $8\frac{1}{2}$ days, where $\frac{1}{2}$ days mean 10–14 hours.

The TEM used for these experiments was dissolved in distilled water. Injections were done subcutaneously after slight ether anaesthesia. The doses administered varied between 1·2 mg. and 2·7 mg. per kg. body-weight, being always divided into three equal parts which were injected on three consecutive days, either on the 5th, 6th, and 7th days (experimental series 1) or on the 7th, 8th, and 9th days of pregnancy (experimental series 2). All doses of TEM were calculated in relation to body-weight, the animals being weighed before each injection. Injections were always carried out at the same time of day. Some of the doses used, especially the largest and the smallest, were administered only in the preliminary experiments for the determination of the most effective dosage. The TEM used was supplied by Imperial Chemicals (Pharmaceuticals) Ltd., Manchester.

In both series of experiments mentioned above the females of the experimental and control groups were usually killed either on the 10th or the 15th day. Some animals, however, were killed between the 15th and 18th day of pregnancy.

A separate group of 72 animals was injected on the 7th, 8th, and 9th day of pregnancy with 1·5 mg. and 1·65 mg. per kg. respectively and kept to term.

Animals were killed by decapitation. Embryos were dissected 3 hours after preliminary fixation in the uterus. Carnoy's fluid and Bouin's solution were used as fixatives. Dissected embryos were embedded in wax for histological examination, serially sectioned, and finally stained with haematoxylin-eosin or methyl green-pyronin. Some 10-day embryos were prepared as whole mounts after staining with Mayer's Haemalum. Fifteen-day embryos were examined histologically and also by observation of their external appearance using × 8 magnification. Some 15-day embryos were also stained with toluidin blue solution and cleared in benzyl benzoate for examination of the skeleton. The external characteristics examined included general body-shape, the straightness of the vertebral column and spinal cord, size and shape of eyes, shape of head, appearance of limbs, shape and number of digits, and appearance of the tail.

To enable the decrease in the developmental rate to be recorded, an approximate scale for the 10-day embryos was used:

1. Control 10-day embryos had 16–21 somites and had completed the turning process (Plate 1, fig. 1). All embryos of the experimental groups whose somite numbers fell within this range were regarded as unaffected as regards developmental rate.
2. Experimental 10-day embryos at the 7–15 somite stage, in the process of turning or immediately after completion (1st degree of retardation—Plate 1, fig. 2).
3. Experimental 10-day embryos at the 3–6 somite stage (2nd degree of retardation—Plate 1, fig. 3).

4. Experimental 10-day embryos at the 1–2 somite stage (3rd degree of retardation—Plate 1, fig. 4).

5. Experimental 10-day embryos with no signs of metamerism of the paraxial mesoderm, at the primitive streak stage, with or without head-fold (4th degree of retardation—Plate 1, fig. 5).

6. Experimental 10-day embryos found in a state of resorption (5th degree of retardation).

The 10-day embryos, both control and experimental, were sectioned longitudinally, as far as possible, and also transversely. The 15-day embryos were sectioned transversely, sagittally and tangentially to the back, depending on what organ system was to be examined.

TABLE 1

*Series 1**

Total dose of TEM (mg./kg. body-wt.)	*Number of females killed on 10th day*	*Number of embryos*		*Number of females killed on 15th day*	*Number of embryos*	
		examined	*dead*		*examined*	*dead*
1·2	8	75	. .	5	42	5
1·35	14	102	2	8	77	6
1·5	24	112	1	8	35	30
1·65	32	211	4	6	28	39
1·8	12	90	23	7	18	57
1·95	11	65	25	7	6	52
2·1	8	48	32	8	4	67
2·25	6	33	28	4	. .	31
2·4	6	28	20	3	. .	28
2·7	7	30	38	. .	. .	. .
Control	18	162	4	15	136	6
Series 2†						
1·2	7	61	3	13	118	4
1·35	15	107	11	19	161	21
1·5	22	174	36	31	149	153
1·65	12	109	14	47	49	446
1·8	7	58	27	12	2	106
1·95	8	57	36	6	. .	59
2·1	6	33	23	4	. .	34
2·25	6	36	30	. .	. .	. .
2·4	6	33	25	. .	. .	. .
2·7	4	18	24	. .	. .	. .
Control	20	184	. .	20	195	6

* Females injected on the 5th, 6th, and 7th day of pregnancy.

† Females injected on the 7th, 8th, and 9th day of pregnancy.

Females belonging to the control groups were likewise anaesthetized on the injection days, without actually being injected.

For comparison of the developmental stages data of Keibel (1937), Snell (1941), and Grüneberg (1943) were used in addition to those obtained from my own control embryos. The paper of Butcher (1929) was very useful for comparing the histology of the somites.

As there was no difference between the control embryos of JU and JC strains, and as the changes following treatment with TEM were the same in both strains, the results from the two strains are reported together.

Quantitative particulars of the animal material are summarized in Table 1.

RESULTS

Series 1

Total doses of 1·2–2·7 mg. per kg. of body-weight were injected on the 5th, 6th, and 7th day of pregnancy (see Table 1, series 1). Each total dose was divided into three equal portions.

10-day embryos. The two smallest doses, i.e. 1·2 mg. and 1·35 mg. per kg. generally did not affect the development of experimental embryos. The 1·35 mg. dose, however, produced in several cases a slight retardation of development. The difference between these and the control embryos did not exceed, on the average, 3–4 somites. Histological examination of the embryonic organs did not show any abnormalities except an occasional and moderate widening of the myocoele in single somites, especially in the 2nd to the 7th somite (Plate 1, figs. 6, 7).

With higher doses (1·5 and 1·65 mg. per kg.) the retardation of development was much more pronounced. The widening of the myocoele was much larger than in embryos of the previous group (Plate 1, fig. 8). The retardation was of the first and second, and in a few cases of the third, degree. In some of the somites an irregularity in shape was observed. They were polygonal in shape with an abnormal outline (Plate 1, fig. 9). Histological examination showed that in embryos treated with 1·65 mg. per kg. disintegration of some somites occurred, especially those in the thorax (Plate 1, fig. 10).

After injection of 1·8 mg. and 1·95 mg. per kg. most of the embryos did not reach stages of development later than that of the primitive streak, i.e. there occurred the 4th degree of retardation. There was also a higher percentage of dead embryos. Primitive streak-stage embryos showed a relatively good development of the head-fold, whereas the paraxial mesoderm did not show any signs of metamerism (Plate 1, fig. 11).

The larger the dose, the greater was the degree of retardation. In general, however, in all these experiments there was considerable variation in the retarding effect of TEM. After the injection of 1·8 and 1·95 mg. per kg. about 20 per cent. and 28 per cent., respectively, of the embryos were being resorbed (5th degree of retardation).

The largest doses (2·1–2·7 mg. per kg.) resulted in a considerably higher percentage of dead embryos, but even after the strongest dose some primitive streak embryos (4th degree of retardation) were still living.

From a comparison of cells in various tissues of 10-day control and experimental embryos it was noticed that, after the injection of 1·35–1·8 mg. per kg., the cells in somites of experimental embryos were slightly larger and with larger nuclei containing 2–3 nucleoli. In somites of the control embryos the majority of the cells contained one nucleolus, and some contained two (Plate 2, figs. 12, 13).

Numerical results of these experiments are shown in Table 2.

TABLE 2

Dose of TEM (mg./kg. body-wt.	*Number of embryos*	*Degrees of retardation*	*Histological characteristics*	*Figs.*
1·2	75	. .	. .	. .
1·35	102	. .	Slight widening of myocoele	7
1·5	112	1, 2, and 3	Pronounced widening of myocoele	8
1·65	211	1, 2, and 3	Widening of myocoele, disintegration of somites	10
1·8	90	4 and 5 (20%)	No metamerism of paraxial mesoderm. Head-folds well developed	11
1·95	65	4 and 5 (28%)	Primitive streak stage	5
2·1	35	4 and 5 (40%)	,,	. .
2·25	22	4 and 5 (46%)	,,	. .
2·4	14	4 and 5 (42%)	,,	. .
2·7	16	4 and 5 (56%)	,,	. .

15-day embryos. The percentage of live embryos on the 15th day of pregnancy, after the same treatment as in the previous group, was comparatively lower. One can assume that some of the retarded embryos still alive on the 10th day had meanwhile died. After the injection of higher doses (1·95–2·4 mg. per kg.) the surviving embryos showed some retardation in development, but there was never more than 24 hours difference between the experimental embryos and the controls. In the whole of this series no particular organic abnormalities were observed except for the rather rare occurrence (5 cases) of a fish-like tail tip (Plate 2, fig. 14). Histological examination of the axial organs did not show any anatomical abnormalities.

After the strongest doses, i.e. after 2·25 mg. and 2·4 mg. per kg., there were no live embryos on the 15th day. The highest dose (2·7 mg.) was therefore abandoned.

Series 2

In this series of experiments TEM was administered on three consecutive days later in pregnancy, i.e. on the 7th, 8th, and 9th day, the total dose being similarly divided into three equal portions injected subcutaneously under slight ether anaesthesia (Table 1, series 2).

10-day embryos. These showed degrees of retardation similar to those of series 1. However, the following differences were observed. The variation in the rate of development after particular dosages, especially that following the injection of 1·5–1·8 mg. per kg., was considerably lower. It was also noticed that, besides the changes in somites previously described, the mid-trunk region of the

5–8 somite stage embryo (at about the 2nd degree of retardation) showed a disproportionally slow development in comparison with the head and caudal regions. Such embryos were markedly shorter than most control embryos at the corresponding stage of development (Plate 2, fig. 15).

Histological examination of the retarded embryos showed that malformation and widening of the somite cavity (myocoele) were frequent. In those embryos which showed shortening of the body the somites in the mid-trunk were in a state of almost complete disintegration, and the somite space was filled with irregularly arranged mesenchyme-like tissue.

TABLE 3

Dose of TEM (mg./kg. body-wt.	*Number of embryos*	*Degrees of retardation*	*Histological characteristics*	*Figs.*
1·2	41	. .	. .	. .
1·35	107	. .	Slight widening of myocoele	. .
1·5	174	2 and 3	Widening of myocoele, disintegration of somites	15, 16
1·65	109	2, 3, and 4	Underdevelopment of the mid-trunk region, oedematous heads	18
1·8	58	4 and 5 (32%)	Primitive streak stage	. .
1·95	57	4 and 5 (35%)	,,	. .
2·1	41	4 and 5 (42%)	,,	. .
2·25	36	4 and 5 (46%)	,,	. .
2·4	43	4 and 5 (43%)	,,	. .
2·7	18	4 and 5 (57%)	,,	. .

General or partial retardation of development was observed according to the dose administered, but extra-embryonic structures seemed not to be influenced so much by treatment with TEM. This was especially true of the allantois and yolk sac, which having been developed much more independently, are presumably less sensitive to the toxic effect of the drug. Many embryos showing the 3rd and 4th degrees of retardation had extra-embryonic structures similar, both in size and structure, to those in control embryos. The allantois in embryos retarded at the primitive streak stage or in the 1–2 somite stage was very long, straight or crooked, extending some distance to the large and spacious yolk sac (Plate 2, figs. 15, 16).

In a few cases, after injecting 1·65 mg. and 1·8 mg. per kg., embryos with pronouncedly oedematous heads and dilatation of brain ventricles were found (Plate 2, figs. 17, 18).

Numerical data for this group of experiments are summarized in Table 3.

15-day embryos. Administration of TEM on the 7th, 8th, and 9th day of pregnancy gave a more precise picture of its action on 15-day embryos. At this time of development it was found that there is a dose of TEM that kills about 50 per cent. of mouse embryos, which are found in a state of resorption in the uterus. This dose is 1·5 mg. TEM per kg., divided into three equal parts administered on the 7th, 8th, and 9th day of pregnancy. This dose may be called 'the embryonic LD_{50}' of TEM. A dose 10 per cent. lower (1·35 mg. per kg.) results in about 90 per cent. survival of embryos, whereas a dose 10 per cent. higher (1·65 mg. per kg.) kills up to 90 per cent. of embryos.

The embryos surviving after administration of 'the embryonic LD_{50}' were always very much retarded in their development, but not by more than 24–36 hours. However, none of the embryos in this group were noticed to have developmental abnormalities.

The next higher dose, i.e. 1·65 mg. per kg., could be regarded as a 'teratological dose' if administered on the same three days. After this dose the majority of the surviving embryos showed changes of various kinds which could be either quantitative alterations in development or even teratological abnormalities.

TABLE 4

Kind of abnormality	*Number of embryos found**	*Percentage*	*General retardation of development in hours*
Kinking or convolution of axial organs (vertebral column and spinal cord)	14	28·5	36–48
Underdevelopment of the eye ball:			
1. unilateral	12	12	24–48
2. bilateral	8	16	36–48
3. coloboma	7	14	36
Brain hernia	4	8	36
Liver hernia	16	32·6	24–48
Underdevelopment of skull and vertebral column resulting in monsters	3	6	48
Shortening of limbs	5	10	36–48
Cauda bifida	13	26	24–36
General underdevelopment only	11	22	24–96

* Some of embryos in this group showed several of above abnormalities and therefore they are recorded more than once in the table.

The general retardation of the developmental rate, if there were no abnormalities observed, was much more severe than in the corresponding experimental groups of series 1. Surviving embryos showed in this case a slowing down of their development amounting to at least 48 hours, and in a few embryos up to 96 hours, compared with 15-day control embryos. Some of them, when examined both macroscopically and histologically, had the same size and appearance as corresponding normally developed 11- to 14-day control embryos. Among them there were, however, many with several developmental abnormalities. Table 4 above summarizes these abnormalities and their frequency.

Kinking and convolution of axial organs. This effect occurred fairly frequently (28·5 per cent.) after administration of the 'teratological dose' (1·65 mg. per kg.). The kinking of the neural tube and of the vertebral column was either one-sided, or in some cases bilateral, resulting in Z-shaped kinking (Plate 2, figs. 19–22). Due to this deformation the body was visibly shortened. Histological examination showed that the kinkage of the axial organs is due to defective metamerism of vertebral column primordia and to irregularities of the spinal ganglia which tend to become fused (Plate 2, figs. 21, 22).

In some cases no real kinking of the vertebral column was observed, but the axial organs showed a slight waviness not found in any of the control embryos.

Underdevelopment of the eyeball. The control embryos of the JC strain, if not albinotic, have a dark pigmented iris shaped as a regular pigment ring (Plate 3, fig. 23). At the embryonic age of 15 days the eyelids are not fused and therefore the eyes are accessible for external examination.

In embryos affected by TEM in teratological dosage, the eyeball was frequently much smaller than normal, being proportionally reduced in all its components. The open eyelids left a smaller opening than usual and the reduced eyeball seemed to be deeper in the socket. The pigment ring in such eyes was irregular in shape or it appeared as a misshapen pigment spot with irregular outline (Plate 3, fig. 24).

Histologically the underdeveloped eyeball consisted of a very small lens surrounded by a relatively large amount of head mesenchyme. Behind this layer the vestigial optic vesicle had a slight invagination at the lens side, which consisted only of a thick layer of the pigment tissue of the retina. Very often the shape of the eye-cup showed that the eye was retarded at a very early stage. The differentiation of all parts of the retina, but not of the pigment layer, seemed to be almost completely retarded, so that the retina was almost non-existent. Strangely enough, the optic nerve in such malformed eyes was often found to be normally developed, being merely smaller than usual in diameter. It was covered with pigment tissue, especially at its distal end, like the control nerves (Plate 3, figs. 25, 26). In these cases penetration of mesenchyme cells and blood-vessels into the choroidal fissure was not observed, whereas it always takes place at that stage in normally developing eyes.

The degree of underdevelopment of the eyeball varied from only a slight diminution to its almost complete absence. The lens, though always present, was very much reduced in size and surrounded by a comparatively large amount of head mesenchyme without being pushed into the secondary optic vesicle.

In some cases, however, the eye appeared to be of normal size but its retinal layers showed a symmetrical concavity at the site of the *papilla nervi optici*. In these cases, although the outside of the eyes looked normal, in histological section it appeared to be almost divided into two parts due to the concavity in the retina. There was, however, one lens only (Plate 3, fig. 27).

Brain hernia. Besides the two cases described under ‘Monsters’ there was observed one embryo with greatly protruding brain tissue causing an eruption on the left side of the front of the head. The brain tissue was outside the brain cavity due to malformation of the skull cartilage in the left frontal region of the skull (Plate 3, fig. 28).

Liver hernia. Besides the normally occurring umbilical hernia in mouse embryos between the 12th and 16th days, the experimental embryos, after the 'teratological' dose of TEM, fairly often (32·6 per cent.) showed a pronounced liver hernia. One or more lobes of the liver protruded outside the adominal cavity, being as a rule covered with a thin, epidermal membrane (Plate 4, figs. 29–31). Often, however, this membrane seemed to be torn off so that the protruding lobes of the liver were completely unprotected. In some cases it should be noted that the opening in the abdominal wall was much smaller than the diameter of the protruding liver lobe.

In experimental embryos kept till term, and also in embryos dissected by Caesarian section on the 21st day of pregnancy, this condition apparently did not improve. It was presumably the cause of death during birth, because many dead embryos were born with liver hernia which left a round opening 2–3 mm. in diameter in the region of the navel.

Monsters. Among all the experimental embryos two 15-day embryos and one new-born mouse were classified as monsters. One 15-day embryo showed pseudencephaly in which the greatly protruding brain was not covered with any skull cartilage. The appearance of this embryo could be compared with mouse embryos described as 'extracranielle Dysencephalie' by Kaven (1938) or as 'pseudencephaly' by Russell (1950*a*). This embryo also showed a unilateral right microphthalmia, a left coloboma, a pronounced one-lobe liver hernia, and 48 hours general underdevelopment (Plate 4, fig. 32).

The second case showed similar pseudencephaly without any cartilaginous skull cover and a complete absence of some primordia of the vertebral column. This condition is known in teratology as pseudencephaly with total rachischisis. In appearance this monster was dominated by a cap of widely protruding brain on its head and a broad, flat, and unclosed plate of the spinal cord with a slightly marked central groove on its back. On the level of the upper thorax the spinal cord plate was deeply kinked towards the inside of the body. Simultaneously this embryo showed a few other malformations like bilateral microphthalmia, shortening of all limbs, a pronounced liver hernia, and about 48 hours general underdevelopment (Plate 4, fig. 33).

Shortening of limbs. This abnormality seems to affect the forelimbs mainly. It was unilateral in three cases, bilateral in two. If unilateral, it is usually correlated with unilateral microphthalmia on the same side of the body, and with kinking of the axial organs directed towards the same side. Shorter limbs did not, however, show any skeletal abnormalities when examined in embryos stained *in toto* with toluidin blue and cleared after dehydration in benzyl benzoate.

Cauda bifida. As a single malformation this was seldom observed, but it was often present with a general underdevelopment of the body. This malformation affected the very tip of the tail, which in some cases appeared to be divided into two parallel outgrowths $\frac{1}{4}$–$\frac{1}{2}$ mm. long (Plate 4, fig. 3). In others it was only a marked flattening of the tail tip resulting in the formation of a fish-like tail tip (Plate 2, fig. 14) similar to those observed in series 1.

General underdevelopment. All the abnormalities described above were accompanied by general underdevelopment as compared with 15-day control embryos. There were also embryos which did not show any morphological abnormalities, but were nevertheless generally underdeveloped. This occurred after all doses of TEM, from 1·35 mg. per kg. upwards. After lower doses, however, and when injections were administered on the 5th, 6th, and 7th day of pregnancy, the retardation did not exceed 36 hours. After the higher doses in series 2, the general retardation of development was much more pronounced and reached as much as 96 hours in extreme cases, although the retarded embryos appeared in all respects to be morphologically normal compared with 11- to 14-day control embryos.

It is worth mentioning that in spite of the pronounced underdevelopment observed in 15-day embryos there was no delay in birth.

DISCUSSION

The toxicity of TEM for mouse embryos, resulting in pre-natal mortality, is a marked characteristic of the drug, especially if it is injected into pregnant females 3–5 days after implantation, i.e. during the formation period of the early embryonic organs. If the injections are done earlier (1–3 days after implantation), when the embryos are not beyond the late primitive streak stage, the toxicity of the drug is less. In surviving 15-day embryos there were almost no malformations, although pre-natal mortality was still high. It can be assumed that early treatment with TEM allows recovery or regeneration of the injured tissues. Similarly, irradiation during pre-implantation stages caused a high percentage of pre-natal mortality, but only a negligible incidence of abnormalities in embryos surviving to term (Russell, 1950*b*).

In amphibian embryos treated with TEM Waddington (1958) observed pronounced selective changes in the neural tube cells, which exhibited dense cytoplasmic aggregates of RNA. In chick embryos the neural tube cells increase in size, as do the nuclei and nucleoli (Jurand, 1958). In the mouse, however, there were no distinct differences in the appearance of the cells in nervous tissue; differences were restricted to somite cells only.

In treated 10-day embryos in series 1 and 2 a progressive development of the head-fold was observed, whereas at that time the paraxial mesoderm did not show any signs of metamerism. Since both head-fold and somites normally make their appearance simultaneously at the $7\frac{1}{2}$-day stage (Snell, 1941) the observed feature can be regarded as an asynchronism in the development of these embryonic organs due to injury to mesodermal activity by TEM.

There is a rather sharply defined toxic dose of TEM (1·5 mg. per kg.) which could be called 'the embryonic LD_{50}', i.e. that dose which kills about 50 per cent.

of embryos when injected subcutaneously. Increasing the dose to 0·55 mg. per kg. per day (total dose, 1·65 mg. per kg.) results in the killing of about 90 per cent. of embryos and causes in the remainder several developmental malformations. These malformations, as well as developmental changes in the metamerism of the paraxial mesoderm, seem to be due to the relatively selective toxicity of TEM for organs originating from the mesoderm. This is confirmed by the kinking of the axial organs in 15-day embryos, this being presumably the consequence of the changes described in the somites in the early stages, which occurred in embryos in series 1 and 2. The previously suggested sensitivity of mesoderm structures to TEM (Jurand, 1958) seems to be confirmed.

Another confirmation of the relative selectivity of TEM is the liver hernia, and probably the brain hernia, which occur in mouse embryos after treatment with the 'teratological dose'. These malformations are presumably due to injury of the mesodermal derivatives taking part in the formation of the abdominal wall and skull cartilage, respectively. At the same time liver and brain, originating from other germ layers, develop at the normal rate, causing an asynchronism which results in excess pressure inside the abdominal cavity and the skull.

More difficult to explain is the mechanism of the abnormal development of the eyeball after TEM treatment. This has been known as a mutation in a strain of mouse and described as anophthalmia (Chase & Chase, 1941). Hertwig (1942) described microphthalmia, a similar condition, due to X-irradiation, but could not decide if it should be regarded as a mutation induced by X-rays. Russell (1950*b*) obtained in mouse embryos microphthalmia and coloboma as the result of irradiation of pregnant females.

The incidence of abnormality of the eyeball is fairly frequent after administration of the 'teratological' dose of TEM. The differentiation of the eye, as generally accepted, involves the induction of the lens and cornea by the developing optic cup, and in any case the optic cup plays the role of the factor responsible for lens development. More recently, however, we find claims that head mesenchyme, or its association with other components of the developing eye, plays an important role in the development of this organ. Holtfreter (1939) has proved that medullary eye-plate deprived of head mesenchyme *in vitro* does not develop beyond a very early stage. His experiments have shown that the eye has a very limited developmental potentiality if regarded as an autonomous unit. Similarly, it has been suggested by Liedke (1951) that in *Amblystoma punctatum* the induction of the lens by the eye-cup takes place only if head mesenchyme is able to make the lens ectoderm respond to the optic cup stimulus. Also Koch & Gowen (1939) have pointed out the failure of mesodermal (pseudoendothelial) tissue in an ophthalmic abnormality which they have described in the mouse.

These suggestions dealing with the role of head mesenchyme may support the following interpretation of the cause of the underdevelopment of the eye described in the present paper. As has been stated, in many cases of eyeball abnormality caused by TEM treatment there is a rudimentary lens surrounded by a comparatively thick layer of head mesenchyme and a vestigial eye-cup with a differentiated pigment layer only. Both lens and eye-cup are fairly close to one another, but apparently their mutual inductive influence is not adequate to ensure full development. It is possible that the more sensitive mesenchyme cells are excluded from the formative process of the eyeball, and that this causes its underdevelopment.

In general, the eyeball underdevelopment induced by TEM treatment can be regarded as a phenocopy, especially of the mutant described by Chase & Chase (1941). The histology of specimens obtained in the present work resembles very closely those described in their paper, particularly those in Plate 2, figs. 20–22.

Malformations induced by TEM, such as cranial defects, anencephaly, and abnormalities of the facial structures were observed by Thiersch (1957) in rat embryos. A short abstract by Nishimura (1957) was also recently published on the influence of TEM on mouse embryos. He found malformations in limbs and jaw, shortening of the tail, inhibition of eyelid development and the splitting of the lips. In a later paper, Kageyama & Nishimura (1959) have shown some malformations of the neural tube and one case of pseudencephaly similar to those described in this paper. The findings of these authors were obtained in embryos kept to term.

SUMMARY

The influence of TEM on mouse embryos has been studied in 10-day and 15-day embryos. Pregnant females were treated on the 5th, 6th, and 7th day and on the 7th, 8th, and 9th day of gestation, respectively.

Ten-day embryos showed, after both variants of TEM administration, a retardation of development which, generally speaking, became more pronounced as the dose increased. A very frequent effect of TEM was the enlargement of the myocoele and, in more severe cases, their disintegration.

If on the 7th, 8th, and 9th days a total dose of 1·5 mg. per kg. was injected in three equal parts, it killed about 50 per cent. of the implanted embryos and was therefore regarded as 'the embryonic LD_{50}'.

A total dose amounting to 1·65 mg. per kg. injected on the same three consecutive days killed about 90 per cent. of the implanted embryos, and the majority of those surviving showed the following developmental malformations: (*a*) kinking and convolution of the axial organs, (*b*) underdevelopment of the eyeball, (*c*) brain hernia, (*d*) liver hernia, (*e*) underdevelopment of skull and backbone resulting in monsters, (*f*) shortening of the limbs, and (*g*) cauda bifida.

ACKNOWLEDGEMENTS

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I would like to thank Professor C. H. Waddington for suggesting this problem, for helpful advice and discussion, and for reading the manuscript. I also wish to thank Dr. D. S. Falconer, Deputy Director of the Agricultural Research Council Unit of Animal Genetics, for the facilities in the mouse house. I am grateful to Miss A. P. Gray and Mr. W. W. Taylor for editorial assistance and to Miss Anne R. Wightman for the photographs.

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EXPLANATION OF PLATES

Plate 1

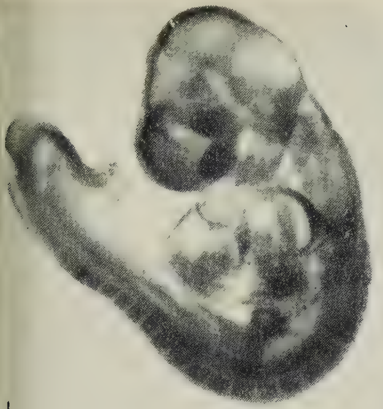
Fig. 1. 10-day control embryo at 20-somite stage. × 30.

Fig. 2. 10-day embryo at 11-somite stage after treatment with 1·5 mg. TEM per kg. body-weight (1st degree of retardation). × 30.

Fig. 3. 10-day embryo at 5-somite stage after treatment with 1·65 mg. TEM per kg. (2nd degree of retardation). × 30.

Fig. 4. 10-day embryo at 2-somite stage after treatment with 1·65 mg. per kg. (3rd degree of retardation).

Fig. 5. 10-day embryo at primitive streak stage after treatment with 1·95 mg. per kg. (4th degree of retardation). × 30.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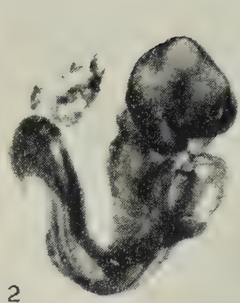
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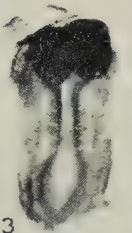
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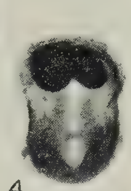
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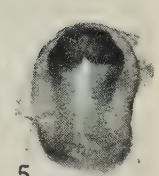
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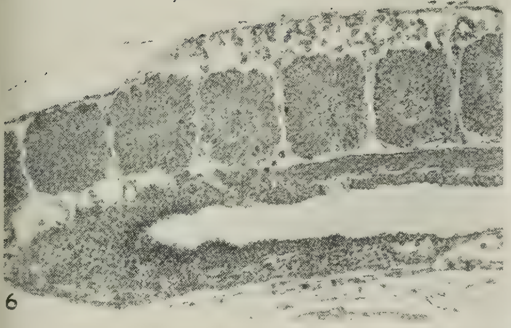
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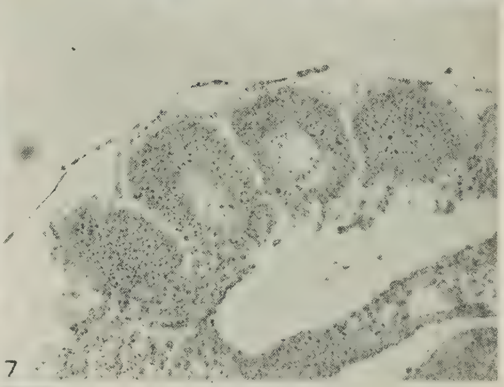
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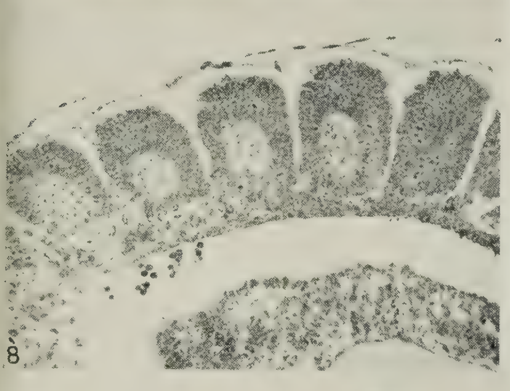
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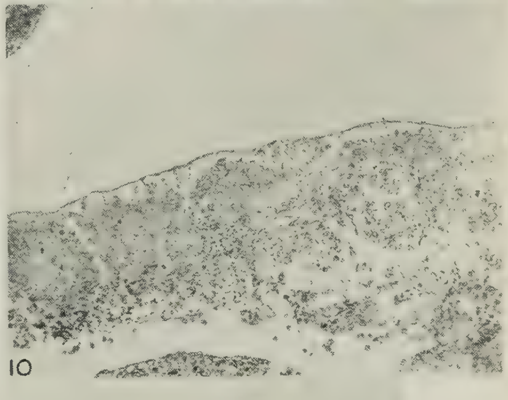
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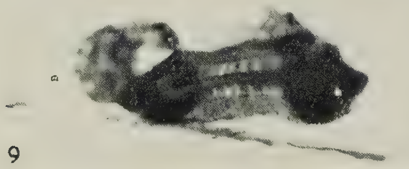
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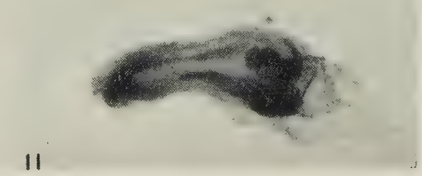
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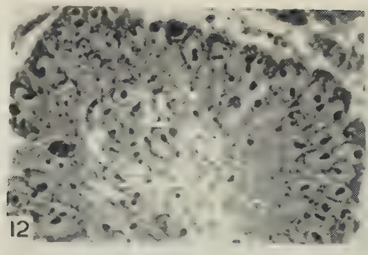
Plate 1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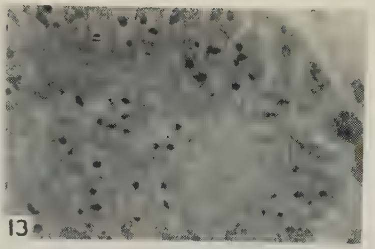
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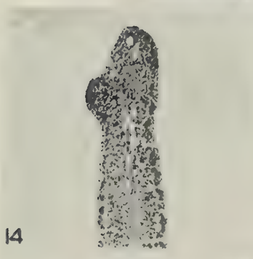
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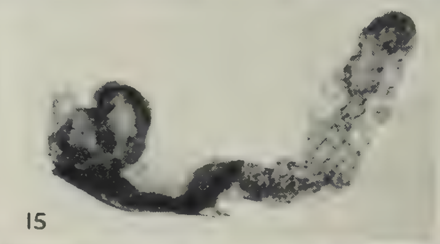
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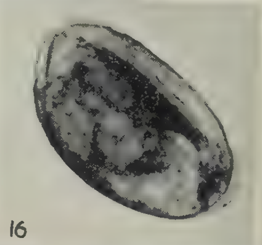
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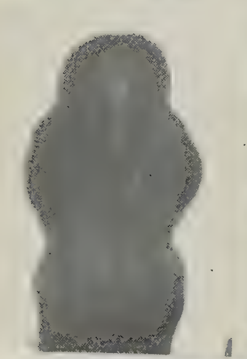
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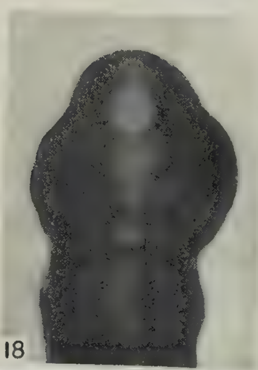
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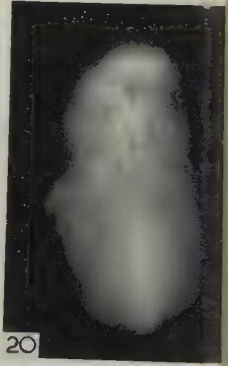
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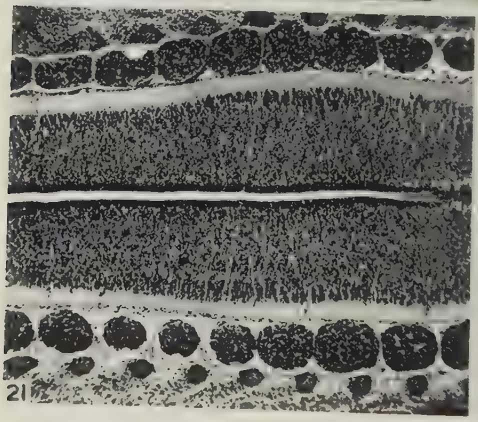
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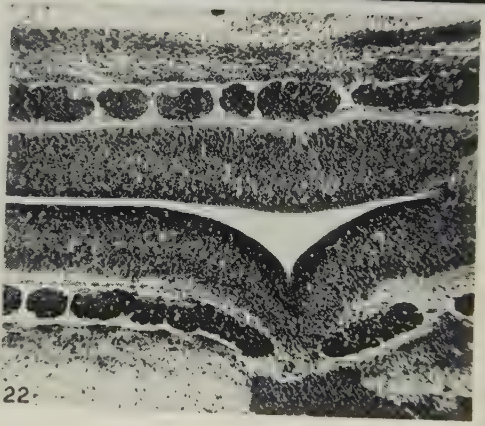
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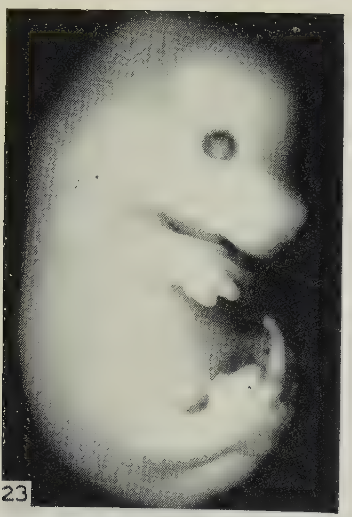

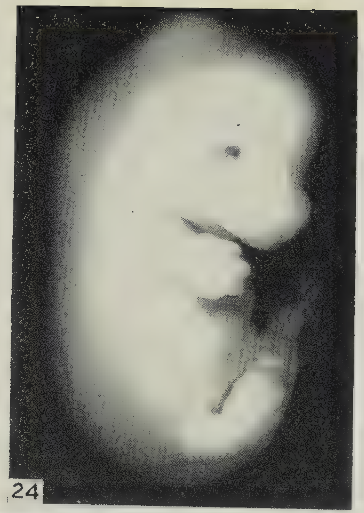

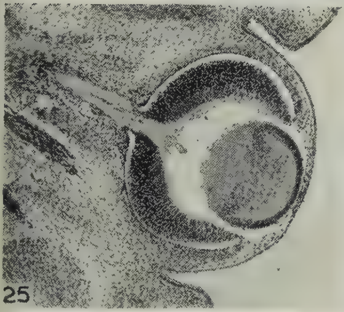

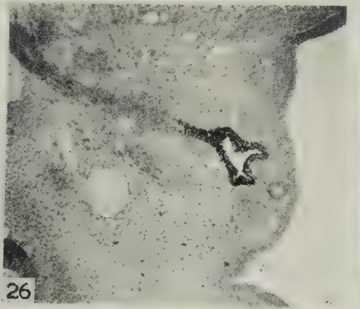

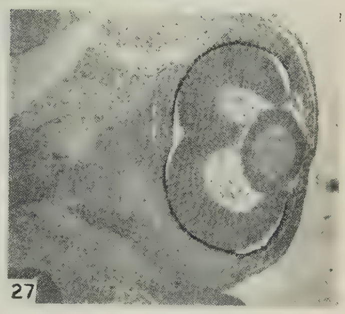

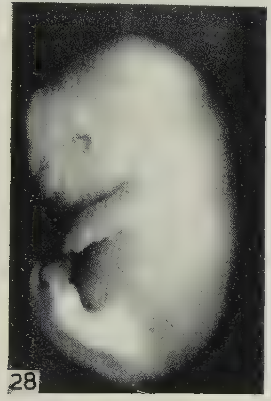

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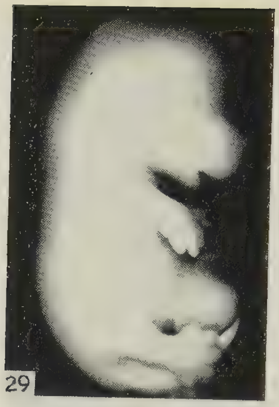
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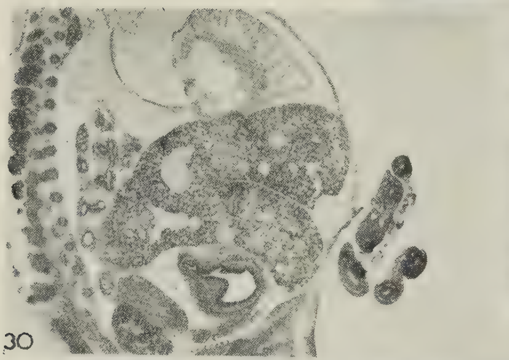
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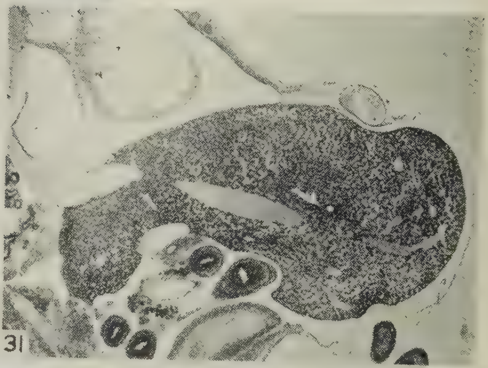
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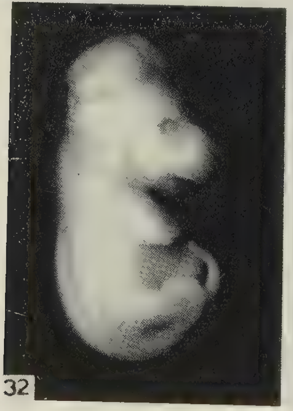
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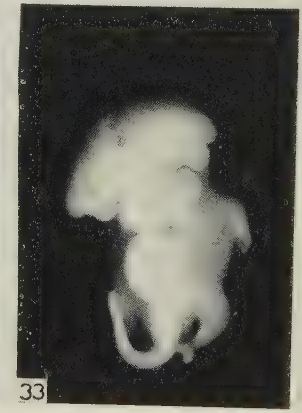
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Plate 4

FIG. 6. Somites of thorax region in 10-day control embryo. × 110.

FIG. 7. Somites of thorax region with moderate widening of myocoele after treatment with 1·35 mg. TEM per kg. × 110.

FIG. 8. Somites of thorax region with very pronounced widening of myocoele after treatment with 1·65 mg. per kg. × 110.

FIG. 9. 10-day embryo showing 2nd degree of retardation with somites of abnormal shape. × 30.

FIG. 10. Progressive disintegration of somites after 1·65 mg. TEM per kg. × 110.

FIG. 11. Primitive streak-stage embryo after treatment with 1·8 mg. per kg. Note well-developed head-fold and no metamerism in paraxial mesoderm. × 30.

PLATE 2

FIG. 12. Cytological picture of a control somite in 10-day embryo. × 380.

FIG. 13. Cytological picture of an experimental somite after treatment with 1·65 mg. per kg. × 380.

FIG. 14. Fish-like tail tip of a 15-day experimental embryo after 1·95 mg. per kg. × 40.

FIG. 15. Experimental 10-day embryo showing 2nd degree of retardation with a shortening of the mid-trunk region but very well-developed head-fold, heart region, and abnormally long allantois. × 30.

FIG. 16. 10-day embryo showing 3rd degree of retardation with a large and spacious yolk sac. × 30.

FIG. 17. Head of a 10-day control embryo. × 30.

FIG. 18. Head of 10-day experimental embryo after 1·65 mg. per kg. Note the dilatation of the brain ventricles (hydrocephalus). × 30.

FIG. 19. Back view of 15-day control embryo. × 4.

FIG. 20. Back view of 15-day experimental embryo with Z-shaped kinking of the axial organs after treatment with teratological dose of TEM. × 4.

FIG. 21. Tangential section of the back through the axial organs of a 15-day control embryo. × 38.

FIG. 22. Tangential section of the back through the kinkage of the axial organs of a 15-day control embryo after treatment with the teratological dose of TEM. × 38.

PLATE 3

FIG. 23. Lateral view of a 15-day control embryo. × 6.

FIG. 24. Lateral view of a 15-day embryo with underdeveloped eyeball after treatment with teratological dose of TEM. × 6.

FIG. 25. Cross-section through the eye of a control 15-day embryo. × 50.

FIG. 26. Cross-section through underdeveloped eye of an experimental 15-day embryo after treatment with the teratological dose of TEM. × 50.

FIG. 27. Cross-section through a malformed eye with concaved retina after treatment with the teratological dose. × 50.

FIG. 28. Experimental 15-day embryo with brain hernia after treatment with the teratological dose of TEM. × 4.

PLATE 4

FIG. 29. Experimental 15-day embryo with liver hernia after treatment with the teratological dose of TEM. × 4.

FIG. 30. Sagittal section through abdominal cavity of a 15-day control embryo. × 20.

FIG. 31. Sagittal section through abdominal cavity and liver hernia of 15-day experimental embryo, after treatment with the teratological dose. × 20.

FIG. 32. 15-day monstrous embryo with anencephaly. × 4.

FIG. 33. 15-day monstrous embryo with anencephaly and rachischiasis. × 4.

FIG. 34. Longitudinal section through the *cauda bifida* in 15-day experimental embryo. × 40.

(Manuscript received 19 : iii : 59)

Recherches sur l'animalisation de l'œuf d'Oursin par les ions Zinc

par R. LALLIER[1]
Station Zoologique de l'Université de Paris, Villefranche-sur-Mer

La différenciation de l'embryon d'oursin peut être modifiée expérimentalement par des agents chimiques. On peut obtenir ainsi la végétativisation des embryons, c'est à dire l'extension des régions entomésodermiques, en traitant les œufs par le chlorure de lithium (Herbst, 1893; Lindahl, 1936). L'animalisation ou extension des territoires ectodermiques est provoquée par les ions sulfocyanure (Lindahl, 1936), l'acide iodosobenzoïque (Runnström & Kriszat, 1952) et l'acide thiomalique (Lallier, 1952). Nous avons montré au cours de recherches antérieures que l'animalisation peut être obtenue aisément en traitant les œufs par les ions zinc (Lallier, 1955 *a*, *b*) ou par certains dérivés organiques acides (Lallier, 1956, 1957, 1958*a*). L'animalisation obtenue dans ces conditions est particulièrement forte et homogène.

Dans ce travail nous étudierons: 1. L'évolution de la sensibilité aux ions zinc au cours du développement embryonnaire. 2. Les interférences entre l'animalisation et la végétativisation au cours des traitements simultanés par les ions zinc et le chlorure de lithium. 3. L'additivité des effets des agents animalisants au cours des traitements simultanés par les ions zinc et un colorant polysulfonique, le bleu Evans. Une note préliminaire a été présentée (Lallier, 1958*b*).

MATÉRIEL ET MÉTHODES

Les œufs de l'oursin *Paracentrotus lividus* sont utilisés dans ces expériences. Les œufs fécondés sont lavés plusieurs fois à l'eau de mer avant d'être transférés dans les milieux de culture. Le volume de chaque culture est de 20 c.c., chacune contenant sensiblement le même nombre d'embryons. La température est de 19° à 20° C.

Pour étudier la sensibilité aux ions zinc au cours du développement, nous nous sommes basés sur les études de Hörstadius (1937) sur la sensibilité des œufs entiers aux ions lithium. Chaque expérience comprend deux séries de cultures, et tous les embryons proviennent de la même femelle. Dans la première série, les œufs sont mis dès la fécondation dans une solution de $ZnCl_2$ 5×10^{-4} M.

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Toutes les 4 heures, des embryons sont prélevés, lavés soigneusement et transférés dans l'eau de mer normale où ils poursuivent leur développement. On prépare ainsi 6 cultures correspondant respectivement à des séjours de 4, 8, 12, 16, 20 et 24 heures dans la solution de $ZnCl_2$.

Dans la seconde série, les embryons sont mis dans la solution de $ZnCl_2$ 5×10^{-4} M, à des intervalles de temps déterminés soit: 0, 4, 6, 8 et 10 heures à partir de la fécondation. Dans chacune de ces cultures, on fait deux prélèvements d'embryons, l'un après 10 heures, l'autre après 24 heures de séjour. Ces embryons sont reportés dans l'eau de mer normale après lavages.

Pour l'étude des traitements simultanés par LiCl et $ZnCl_2$, la concentration de $ZnCl_2$ fixée à 5×10^{-4} M reste constante, tandis que la concentration de LiCl varie de $6{,}5 \times 10^{-3}$ M à 26×10^{-3} M. Les œufs sont traités à partir de la fécondation pendant 22 heures consécutives puis reportés, après lavages, dans l'eau de mer normale.

Pour l'étude de l'additivité des effets de deux agents animalisants, nous utilisons le bleu Evans à la concentration 5×10^{-6} M et $ZnCl_2$ aux concentrations 5×10^{-6} M et 10^{-5} M. Des sels de zinc et de lithium pour analyse sont utilisés pour la préparation des solutions. La solution stock de $ZnCl_2$ M/500 dans l'eau de mer est titrée par la méthode de Vosburg, Cooper, Clayton, & Pfann (1938). La solution stock de LiCl 0,65 M est préparée dans l'eau distillée. Le bleu Evans provient des Laboratoires Eastman Kodak. Les dilutions de ce colorant sont faites à partir d'une solution stock 2×10^{-4} M dans l'eau de mer.

RÉSULTATS

Evolution de la sensibilité aux ions zinc au cours du développement

Examinons d'abord les effets de traitements de durées différentes commencés dès la fécondation. Les résultats sont présentés dans la fig. 1.

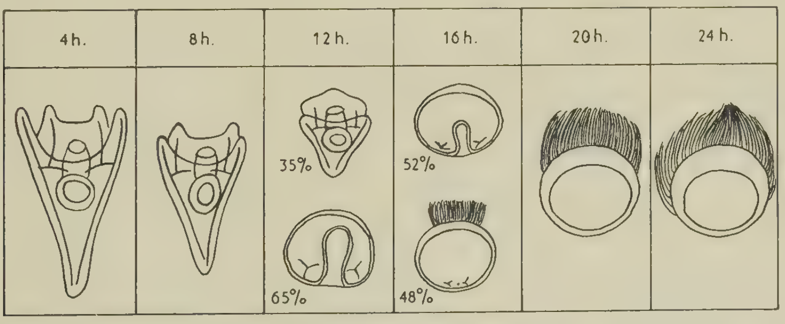

FIG. 1. Action du chlorure de zinc sur l'œuf entier. Les œufs sont mis dès la fécondation dans la solution de chlorure de zinc et y sont laissés respectivement pendant 4, 8, 12, 16, 20 et 24 heures.

On remarque que dans ces conditions l'animalisation n'est pas obtenue après 8 heures de traitement. Les plutei sont seulement plus petits que les témoins, cet effet est plus marqué après 8 heures de séjour. L'animalisation ne s'observe qu'avec des traitements de 16 heures. Elle est toutefois peu prononcée et n'atteint que la moitié des embryons. L'animalisation est forte et homogène dans les cultures traitées pendant 20 à 24 heures consécutives. Les pontes provenant de femelles différentes présentent des variations de sensibilité, mais toutes les expériences montrent la même augmentation de l'effet animalisant avec l'allongement de la durée du traitement. Ces variations de sensibilité ne s'observent d'ailleurs que pour les traitements de courte durée. A partir de 20 heures de traitement l'animalisation est uniforme pour toutes les pontes.

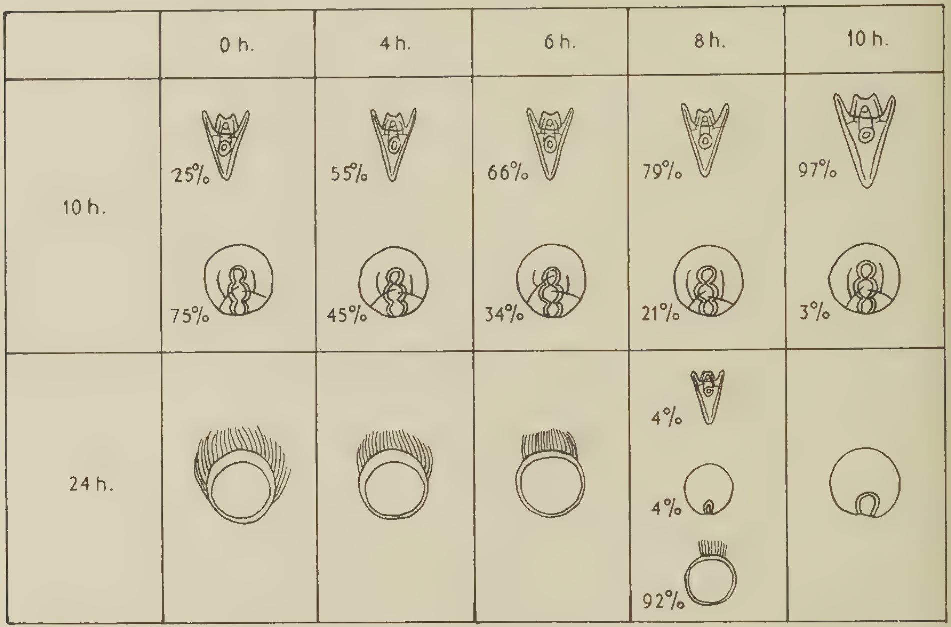

Fig. 2. Action du chlorure de zinc sur l'œuf entier. Les œufs sont mis 0, 4, 6, 8 et 10 heures après la fécondation dans la solution de chlorure de zinc. Dans la rangée supérieure sont représentés les embryons traités pendant 10 heures consécutives et dans la rangée inférieure les embryons traités pendant 24 heures.

Examinons maintenant les effets de traitements commencés à des stades différents du développement et prolongés respectivement pendant 10 et 24 heures. Les résultats sont présentés dans la fig. 2.

Deux constatations ressortent de cet examen. (*a*) Les traitements de 24 heures sont plus efficaces que ceux de 10 heures. Les traitements de 24 heures permettent d'obtenir une extension caractéristique de la touffe ciliée apicale, qui n'est pas obtenue avec les traitements de 10 heures. (*b*) Pour une même durée de traitement l'animalisation est d'autant plus forte que le traitement est commencé plus tôt après la fécondation. Les observations précédentes concernant les variations de la sensibilité selon les pontes s'appliquent également ici.

Traitements simultanés par LiCl et $ZnCl_2$

Les résultats sont présentés dans la fig. 3. Les embryons, après 24 heures de culture, sont lavés et reportés dans l'eau de mer normale où ils achèvent leur développement. La végétativisation est très prononcée dans les embryons cultivés en présence de $ZnCl_2$ 5×10^{-4} M et LiCl $19{,}5 \times 10^{-3}$ M. Les effets animalisants du zinc ne se manifestent pas dans ces conditions où la concentration en ions Li est 39 fois celle des ions Zn. En diminuant la concentration en ions Li jusqu'à 13×10^{-3} M, on observe de très nets changements dans la morphologie des larves. Les embryons sont en majorité végétativisés mais de façon plus discrète et on note la présence de plutei de structure normale mais plus petits que les plutei témoins. Dans ces cultures la concentration en ions Li n'est plus que 26 fois celle des ions Zn. Lorsque la concentration en LiCl s'abaisse à $6{,}5 \times 10^{-3}$ M les petits plutei augmentent en nombre cependant que l'on note la présence d'embryons animalisés avec une touffe ciliée apicale élargie correspondant au type 1/4 selon la terminologie de Hörstadius (1935) ainsi que des embryons animalisés à un degré plus faible. Dans ces conditions la concentration en ions Li est 13 fois supérieure à celle des ions Zn.

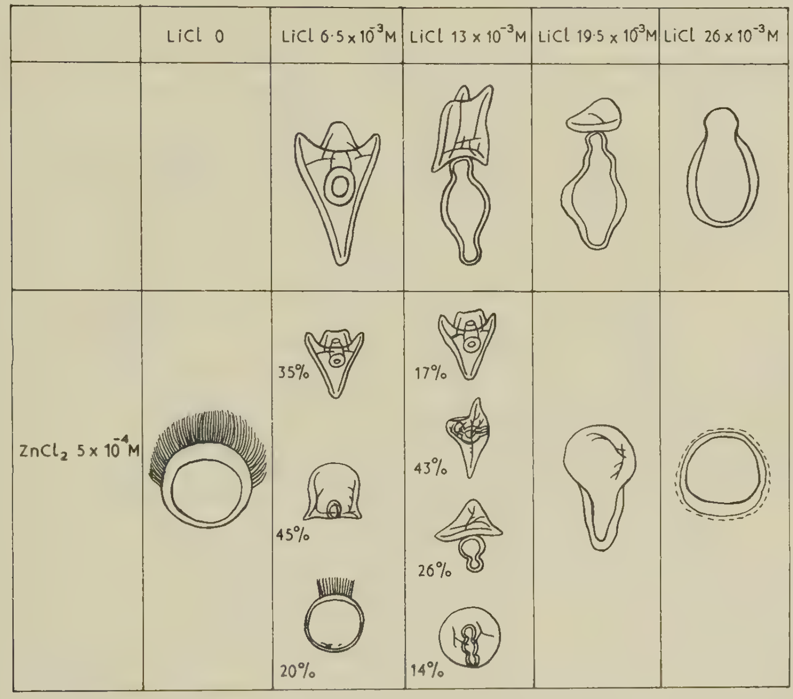

FIG. 3. Traitements simultanés des œufs entiers par le chlorure de zinc et le chlorure de lithium. Dans la rangée supérieure sont représentés les embryons traités par le chlorure de lithium à différentes concentrations. Dans la rangée inférieure sont représentés les embryons traités par le chlorure de zinc en présence de concentrations croissantes de chlorure de lithium.

Traitements simultanés par $ZnCl_2$ et le bleu Evans

Les résultats sont présentés dans la fig. 4. Utilisé seul aux concentrations 10^{-5} M et 5×10^{-6} M, $ZnCl_2$ induit le développement de formes larvaires à symétrie radiale. Il en est de même avec le bleu Evans à la concentration 5×10^{-6} M. Agissant ensemble à ces faibles concentrations $ZnCl_2$ et le bleu Evans provoquent l'animalisation des larves et celles-ci ne présentent plus qu'une ébauche d'archentéron.

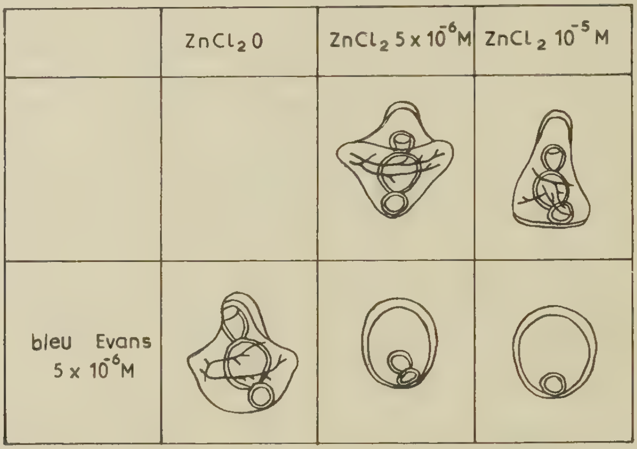
$ZnCl_2$ 0
$ZnCl_2$ 5×10^{-6} M
$ZnCl_2$ 10^{-5} M
bleu Evans 5×10^{-6} M

FIG. 4. Traitements simultanés des œufs entiers par le chlorure de zinc et le bleu Evans. Dans la rangée supérieure sont représentés les embryons traités par le chlorure de zinc à différentes concentrations. Dans la rangée inférieure sont représentés les embryons traités par le bleu Evans en présence de concentrations croissantes de chlorure de zinc.

DISCUSSION

L'étude de l'action des ions Zn au cours du développement embryonnaire montre que les effets animalisants diminuent d'intensité avec la progression du développement. Les œufs doivent être traités dès les premiers stades de la segmentation pour que les ions Zn exercent leurs effets animalisants au maximum. Avec le lithium également, la sensibilité maximum se situe au cours des premiers stades de la segmentation ainsi que l'ont montré Herbst (1893), Hörstadius (1937), Lindahl (1940), Backström & Gustafson (1953). Si l'on examine maintenant l'évolution de la sensibilité aux agents animalisants, on note que l'acide iodosobenzoïque comme les ions Zn est plus efficace lorsqu'il agit sur les premiers stades du développement. Un traitement prolongé renforce son action (Backström, 1953). Par contre avec le sulfocyanure de sodium, l'animalisation est plus efficacement obtenue en traitant les œufs avant la fécondation. Les œufs traités après la fécondation montrent une combinaison d'effets animalisants et d'effets inhibiteurs de la différenciation (Lindahl, 1936). Le traitement des œufs avant la fécondation par les ions Zn provoque des perturbations de la fécondation et de la segmentation (Cleland, 1953). En raison de l'évolution parallèle de la sensibilité aux ions Zn et Li, il était intéressant d'étudier les effets d'un traitement simultané par ces deux agents, afin d'examiner dans quelle mesure leurs effets sur la détermination embryonnaire peuvent se corriger mutuellement. Nous ferons tout d'abord remarquer que ces agents agissent à des concentrations très différentes. Pour des effets végétativisants et animalisants très accentués, les concentrations sont de l'ordre de 26×10^{-3} M pour les ions Li et seulement de 5×10^{-4} M pour les ions Zn. Dans ces conditions les ions Zn sont actifs à une concentration 50 fois plus faible que celle des ions Li. Dans les cultures mixtes étudiées nous avons observé le développement d'un petit pourcentage de plutei de structure normale lorsque la concentration en ions Li est environ 10 à 20 fois supérieure à celle des ions Zn. La compensation des effets animalisants du zinc et des effets végétativisants du lithium n'est toutefois pas parfaite puisque, d'une part elle ne s'observe que pour un petit pourcentage d'embryons et que, d'autre part, ceux-ci restent plus petits que les témoins. Une conclusion analogue avait été présentée à la suite d'une étude effectuée précédemment dans des conditions légèrement différentes (Lallier, 1955*b*). La suppression de l'animalisation par le lithium a également été observée avec d'autres agents animalisants, notamment le sulfocyanure (Lindahl, 1936) et la trypsine (Moore, 1952).

Nous avons examiné dans un travail antérieur (Lallier, 1957) les effets du chlorure de lithium sur l'animalisation par le bleu de ciel (chlorazol sky blue). Dans ces conditions, les embryons passent des formes végétativisées typiques pour des concentrations suffisantes en chlorure de lithium, aux formes animalisées lorsque la concentration en chlorure de lithium diminue. Mais on n'observe pas le développement de formes plutéiques. La compensation des effets animalisants par le lithium apparaît ainsi plus difficile avec ce colorant polysulfonique que dans le cas des ions zinc. Ces différences peuvent être mises en relation avec le mode d'action de ces agents et en particulier avec leur affinité pour les protéines.

Ranzi (1951) a montré que les ions Li provoquent des changements dans la structure des molécules protéiques. Ceux-ci peuvent à leur tour modifier l'affinité des protéines pour les ions Zn notamment. Une diminution de l'affinité pour ces ions entraînerait un affaiblissement de l'effet animalisant. Des colorants polycycliques acides, tels que le bleu de ciel ou le bleu Evans, se fixent aux groupes basiques des protéines par leurs groupes sulfoniques. La stabilité des complexes ainsi formés peut être augmentée par l'établissement de liaisons de type Van der Waals entre les noyaux aromatiques du colorant et les protéines. Cette augmentation de stabilité peut rendre compte d'une part de la grande activité animalisante de ces dérivés polycycliques acides, ceux-ci agissant en effet à des concentrations très faibles, et d'autre part de l'impossibilité d'obtenir une compensation complète lors d'un traitement simultané par LiCl.

Une interprétation des effets radialisants et animalisants des dérivés polysulfoniques et des ions Zn peut être présentée. La radialisation s'exprimerait au dessous d'un certain seuil de fixation de l'agent et l'animalisation apparaîtrait lorsque la quantité d'agent fixé dépasse ce seuil. Cette conception peut rendre compte de l'additivité des effets des ions Zn et du bleu Evans.

Les ions Zn ont une affinité marquée pour les groupes imidazoles des protéines (Gurd & Goodman, 1952). Ces groupes en raison de leur caractère basique sont également capables de fixer les dérivés organiques acides. Ils ne peuvent toutefois être considérés comme exclusivement responsables de la fixation de ces agents animalisants. L'étude de la fixation des agents animalisants sur les protéines et des conséquences qui en découlent au point de vue de la synthèse des protéines spécifiques au cours du développement apparaît ainsi essentielle pour la compréhension du déterminisme des modifications expérimentales de la détermination embryonnaire.

RÉSUMÉ

1. Les effets animalisants des ions zinc sur le développement de l'œuf de l'oursin *Paracentrotus lividus* sont étudiés.

2. La sensibilité des embryons aux ions zinc est plus forte au début du développement. L'animalisation obtenue est plus forte lorsque le traitement par les ions zinc est prolongé au delà du stade blastula. Les sensibilités aux ions zinc et aux ions lithium au cours du développement évoluent parallèlement.

3. Les effets d'un agent végétativisant, le chlorure de lithium, sur l'animalisation par les ions zinc, sont étudiés sur des cultures mixtes en faisant varier la concentration en ions lithium, la concentration en ions zinc restant fixe. Les embryons passent du type végétativisé au type animalisé lorsque la concentration en ions lithium diminue. Quelques plutei de structure normale mais de petite taille peuvent être obtenus avec une concentration convenable en ions lithium.

4. Les ions zinc à la concentration 5×10^{-6} M et 10^{-5} M et le bleu Evans, un colorant polysulfonique acide, à la concentration 5×10^{-6} M induisent le développement de larves à symétrie radiale. Agissant ensemble à ces concentrations, ces agents exercent un effet animalisant.

5. Le déterminisme de l'animalisation de l'œuf d'oursin par les ions zinc est discuté en fonction de leur affinité pour les protéines et des interférences qui en résultent avec la synthèse des protéines spécifiques au cours du développement embryonnaire.

SUMMARY

1. The animalizing effects of zinc ions on the development of eggs of the sea-urchin *Paracentrotus lividus* were studied.

2. The susceptibility of the embryos to zinc ions is stronger at the beginning

of development. Treatment by zinc ions has to be extended beyond the blastula stage in order to get the animalizing effects maximally expressed. The evolution of susceptibility to zinc ions during development is similar to the evolution of susceptibility to lithium ions.

3. The action of a vegetalizing agent, such as lithium chloride, on the animalizing effects of zinc ions was studied in cultures with zinc chloride at a concentration of 5×10^{-4} M and lithium chloride at concentrations varying over a fairly wide range. With decrease in concentration of lithium chloride, the embryos pass from vegetalized to animalized types. Very small plutei can be obtained with suitable concentrations of lithium chloride.

4. Both zinc ions at concentrations of 5×10^{-6} M and 10^{-5} M, and the acid polysulfonic dye, Evans blue, at a concentration of 5×10^{-6} M, induce the development of radial larvae. Acting together at these concentrations, these agents exert an animalizing effect. The summation of the effects of these two animalizing agents is thus demonstrated.

5. The process of animalization by zinc ions in the sea-urchin egg is discussed in relation to the affinity between animalizing agent and proteins, and the interference with synthetic processes of specific proteins during embryonic development.

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An Immunological Approach to the Problem of Lens Regeneration

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WITH ONE PLATE

INTRODUCTION

LENS regeneration from the dorsal rim of the iris has been observed after removal of the original lens in many species of the genus *Triturus* (Stone, 1952, 1954, 1958; Zalokar, 1944; Reyer, 1954). Transformation of the iris cells into lens starts with depigmentation and is followed by multiplication of the cells, which become arranged into a vesicle. Subsequently the cells in the posterior wall of the vesicle differentiate into lens fibres and, finally, a new lens is formed. Similarly in the chick (Van Deth, 1939, 1940) removal of the lens primordium from a 53-hour embryo and explantation of the eye-cup resulted in formation of a small lens from both iris epithelium and pigment layer of the retina. However, in the chick the lens-forming potency of the iris was not limited to the dorsal part, but extended also to the ventral rim.

A biochemical relationship between the lens and such lens regenerating tissues as iris and retina was suggested by data obtained with immunological techniques. By injecting lens antiserum into chick embryos, Burke, Sullivan, Peterson, & Weed (1944) observed cytotoxic effects not only in the lens epithelium, but also in the pigment layer of the retina, thus suggesting the presence of lens antigens in retina cells. By labelling lens antibodies with fluorescent dyes, Clayton (1954) observed fluorescence both in the lens and in the ciliary process and retina of 7-day mouse embryos. Similarly, I^{131}-labelled antiserum made it possible to trace lens antigens in the lens, the retina, the epithelial layer of the cornea, the sclera and in the extrinsic eye muscles and ciliary muscle (Clayton & Feldman, 1955). Since the lens tissue used for immunization was not adequately freed of surrounding tissues (Clayton, 1954) the possibility that the reactions were partly due to tissue contamination cannot be excluded. The experiments of Ten Cate & Van Doorenmaalen (personal communication), Langman, Schalekamp, Kuijken, & Veen (1957), and Van Doorenmaalen (1958), in which the presence of lens antigens was demonstrated in extracts of iris and retinal tissue using

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Boyd's (1956) precipitin technique, also did not exclude the possibility of contamination with lens material.

Since the presence of lens antigens in iris and retina may be related to the ability of these tissues to regenerate new lenses after lentectomy, it was thought necessary to establish beyond doubt whether the lens antigens found in iris and retina were components proper to these tissues or whether they were due to contamination with lens material. The present work was therefore undertaken to study the presence of lens antigens in various tissues of the chick under conditions excluding the possibility of contamination by lens components, by using tissues of eyes previously lentectomized. In addition, the number of lens antigens present in the various tissues of the eye and their relationship to antigens of the lens proper was studied.

MATERIALS AND METHODS

Antibody preparation

Adult chicken lenses were removed and under the dissecting microscope carefully cleaned from surrounding eye tissues. Whenever it was impossible to remove small adherent parts of the iris, the lens was discarded. The lens tissue was weighed and homogenized to a concentration of 100 mg. wet weight/ml. in sterile 0·9 NaCl solution. This extract was administered to adult albino rabbits intravenously at the rate of one injection of 3 ml. on alternate days for 14 days. Ten days after the last injection the rabbits were bled and the serum filtered through a Seitz filter. The titre of the antiserum was determined by testing it against serial dilutions of 10 per cent. chicken lens extract and found to vary from 1:6,400 to 1:16,000.

Antigen preparation

The left eyes of young adult chickens were lentectomized and the removed lenses carefully studied under the dissecting microscope. Whenever the lens capsule was found ruptured, indicating that parts of the lens or lens capsule were possibly left behind in the eye, the animal was not used for the test. Six to seven weeks after the lentectomy the animals were killed and 20 per cent. saline extracts were prepared of the following tissues from the right (lens containing) eyes and separately from the left (lensless) eyes: dorsal and ventral rim of the iris, pigment and neural layer of the retina, cornea, sclera, and vitreous body. Aqueous humour was obtained in undiluted form. Similarly, 20 per cent. saline extracts were prepared from the following tissues of normal chicken: brain, skin, comb, lung, liver, pancreas, stomach, spleen, bladder, skeletal muscle, heart muscle, kidney, and ovary.

Antigen-antibody reaction

Serial dilutions of the tissue extracts were tested with lens antiserum in micro test-tubes according to Boyd's (1956) precipitin technique, modified by Ten Cate

& Van Doorenmaalen (1950). The extracts which gave a positive ring test were subsequently tested with lens antiserum in the double diffusion technique of Ouchterlony (1949, 1953). This technique is based on the fact that different antigens diffuse in agar at different speed. Precipitin bands are formed in the agar when critical concentrations of the diffusing antigens and their corresponding antibodies meet. Agar plates were prepared with 20 per cent. dialysed filtered agar (Difco —B 140; pH 7·2) to which 0·01 per cent. merthiolate (Eli Lilly & Co., solution No. 45) had been added. Ten ml. of this solution (at 85° C.) were pipetted into Petri dishes to cover the slightly curved bottoms and allowed to solidify. Another 15 ml. of agar was poured over this layer and two Plexi-glass moulds were placed in the warm solution 6–12 mm. from each other. After the agar had solidified the moulds were removed, thus leaving two wells referred to as the 'central' well (10 × 10 × 3 mm.) and the 'peripheral' well (10 × 5 × 3 mm.). The central well was filled with lens antiserum and the other with tissue extract. Similarly, agar plates were prepared in which a circular central well (diam. 12 mm.) and eight circular peripheral wells at a regular distance from each other, and 10 mm. from the central well, were fashioned in the solidifying layer. In this way it was possible to test extracts of several tissues with the same lens antiserum in one single agar plate. The agar plates were examined daily and the appearance of precipitin bands recorded.

RESULTS

Boyd's precipitin test showed the presence of lens antigens in the following tissues obtained from lentectomized eyes: dorsal and ventral parts of the iris and pigment layer of the retina and cornea. Their presence in vitreous body and aqueous humour was doubtful, while no lens antigens were found in any of the extra-ocular tissues (Table 1). Extracts prepared from tissues of lens containing eyes showed exactly the same results.

TABLE 1

Precipitin reaction with lens antibodies

Tissue	*Reaction*	*Tissue*	*Reaction*
Dorsal iris	++	Cornea	+
Ventral iris	++	Vitreous body	±
Pigment retina	+	Aqueous humour	±
Nervous retina	–	Sclera	–
Brain	–	Bladder	–
Skin	–	Skeletal muscle	–
Comb	–	Heart muscle	–
Lung	–	Kidney	–
Liver	–	Ovary	–
Pancreas	–	Blood-serum	–
Stomach	–	Spleen	–

KEY: – Negative ring test. ± Doubtful ring test. + Positive ring test (titre 1 : 80). ++ Positive ring test (titre 1 : 640).

When the positive reacting tissues, including the lens, were tested with lens antiserum according to the agar diffusion method of Ouchterlony, several precipitin bands became visible between the peripheral and central well. The number of bands, indicating the minimum number of lens antigens present in the tissues, varied from 2 to 7, depending on the tissue tested (Table 2, Plate, figs. 1–6). The number and location of the precipitin bands was exactly the same whether or not the lens had been removed 6–7 weeks prior to the test. None of the other tissues showed the formation of precipitin bands when tested with lens antiserum in the agar plate.

TABLE 2

Tissue	*Concentration*	*Number of bands*	*Fig. of Plate*
	(%)		
Lens	10	6–7	1
Iris	20	4	2
Cornea	20	1–2	3
Vitreous body	20	3	4
Pigment retina	20	4	5
Aqueous humour	100	4	6

Number of precipitin bands formed by testing eye tissue extracts with lens antiserum according to Ouchterlony's agar diffusion test.

When extracts of the various positive reacting eye tissues were tested with lens antiserum in a single agar plate, one to six precipitin bands, the number depending on the source of tissue tested, became visible between the central and peripheral wells. By daily recordings it was possible to establish the relationship between the 6 precipitin bands formed by the lens and each of the bands caused by the other tissues, since precipitin bands from two adjacent wells merge in case of identity (Table 3, Plate, fig. 7). No crossing of bands or spur formation, indicating non-identity of antigens, was observed during the test period.

TABLE 3

	Precipitin bands					
Tissue	*1*	*2*	*3*	*4*	*5*	*6*
Lens	+	+	+	+	+	+
Pigment retina	+	+	+	+		
Iris			+		+	+
Cornea		+				
Vitreous body		+		+	+	+
Aqueous humour	+		+		+	+

Merging of precipitin bands, formed by the various eye tissues, with one or more of the 'lens' bands, indicated by a +.

DISCUSSION

In the double diffusion technique of Ouchterlony (1949, 1953) antigens and antibodies are placed in opposite wells and diffuse into 'neutral' agar. Where an antigen and its corresponding antibody meet in critical concentration, a narrow band of precipitate is formed. Neither antibody nor antigen can diffuse beyond this precipitin line. Unrelated antigens and antibodies, however, will not be disturbed in their diffusion by this barrier (Ouchterlony, 1949, 1953; Wilson & Pringle, 1955; Korngold, 1956). When a number of antigens and antibodies are used, a precipitin spectrum develops, each band corresponding to an antigen-antibody pair. Extracts of different tissues placed in adjacent peripheral wells and tested against the same antiserum may show merging precipitin bands, indicating identity of antigenic components causing the bands. Crossing of bands or 'spur formation' indicates non-identity of antigenic components.

In this experiment a 10 per cent. aqueous lens extract prepared from adult chicken showed the formation of 6 to 7 distinct precipitin bands, when tested with lens antiserum (Plate, fig. 1). Therefore, the lens of young adult chicken contains substantial amounts of at least 6 to 7 water-soluble antigenic components (Langman, 1959*a*, 1959*b*). The possibility, however, exists that more antigens were present but remained undetected by the method used.

Extracts of iris, pigment layer of the retina, cornea, vitreous body, and undiluted aqueous humour tested with lens antibodies formed 2 to 5 precipitin lines (Plate, figs. 2–6), indicating the presence of 2 to 5 lens antigens in the various tissues. Contamination of these tissue extracts by lens antigens can be excluded, since extracts prepared from eyes in which the lens had been removed 6–7 weeks prior to the test yielded the same number of bands as extracts prepared from lens containing eyes. Whenever small remaining parts of the lens were detected at the time of dissection of the tissues from lentectomized eyes, the eyes were discarded. The precipitin bands found by testing the various eye-tissue extracts with lens antiserum are therefore caused by antigens proper to the tissues tested and in some way incorporated in the cells.

The fact that the precipitin bands caused by iris, pigment layer of the retina, cornea, aqueous humour, and vitreous body merge with some of the bands caused by the lens itself (Table 3, Plate, fig. 7), indicates that these tissues have one or more antigenic components identical with lens antigens. This suggests that under normal conditions antigens identical with lens antigens are built by the cells and integrated into their structure. Under abnormal conditions, such as removal of the original lens in the embryo, the normal equilibrium in the cells of iris, pigment layer of the retina, and cornea may be disturbed and the lens antigens may become building-stones for the formation of lens cells. Indeed, pigment layer of the retina, iris, and cornea, which contain antigens identical with those of the lens, have the ability to form a lens when the original lens is removed early in development (Van Deth, 1939, 1940; Langman, unpublished).

Though the presence of lens antigens in iris, pigment layer of the retina, and cornea can be demonstrated at the young adult stage, the lens regeneration potency of these tissues seems to be restricted to the early embryonic and newborn stage (unpublished experiments).

SUMMARY

1. Application of Boyd's precipitin test showed the presence of lens antigens in extracts of pigment layer of the retina, ventral and dorsal parts of the iris, cornea, vitreous body, and in aqueous humour obtained from eyes in which the lens was removed 6–7 weeks prior to the test. Their presence was not detected in the neural layer of the retina, sclera, or in any other extra-ocular tissue (Table 1). Extracts prepared from lens-containing eyes showed exactly the same results.

2. The number of lens antigens in these tissues was determined by the agar diffusion technique of Ouchterlony and was found to vary from 2 to 7, depending on the source of tissue used (Table 2, Plate, figs. 1–6).

3. The antigens found in pigment layer of the retina, iris, cornea, vitreous body, and aqueous humour appeared to be identical to antigens found in the lens (Table 3, Plate, fig. 7).

4. Since iris, pigment layer of the retina, and cornea have the property to regenerate a lens after removal of the original lens, the obtained data suggest that there is a relationship between the lens regeneration capacity and the presence of antigens identical to those of the lens.

ACKNOWLEDGEMENTS

I wish to thank Dr. C. P. Leblond for his helpful suggestions in the preparation of this paper and Miss S. Clerk and Mr. C. Schneider for technical assistance in the experiments. The work was supported by grants from the National Cancer Institute of Canada and the National Research Council of Canada.

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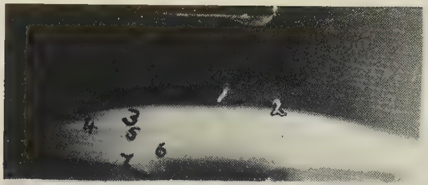

FIG. 1
LENS

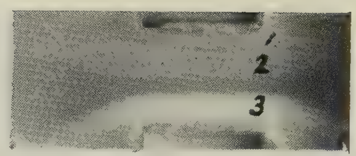

FIG. 4
VITREOUS BODY

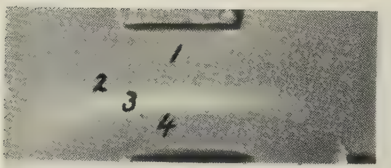

FIG. 2
IRIS

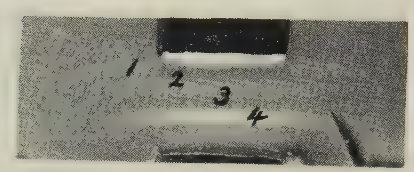

FIG. 5
PIGMENT RETINA

FIG. 3
CORNEA

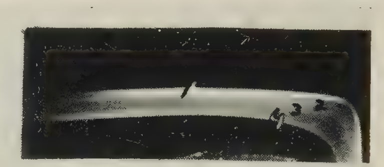

FIG. 6
AQUEOUS HUMOR

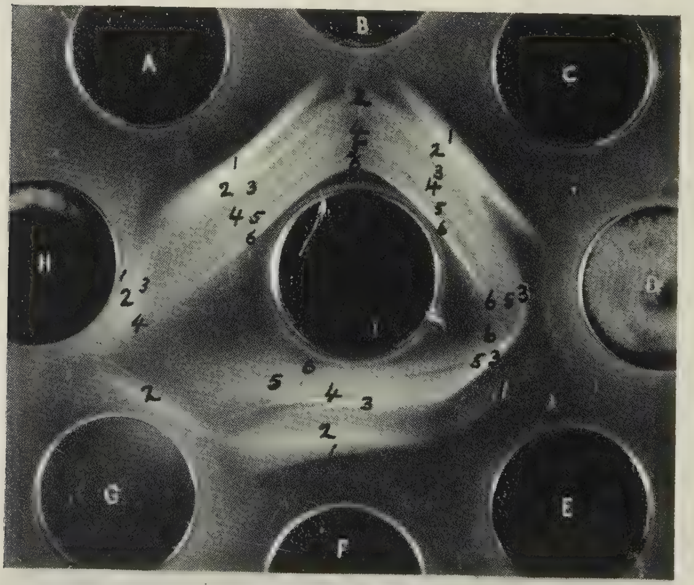

FIG. 7

J. LANGMAN *and* B. D. PRESCOTT, Jr.

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EXPLANATION OF PLATE

FIGS. 1–6. Precipitin bands formed by testing tissue extracts (top well) with lens antiserum (bottom well).

FIG. 7. Precipitin bands formed by testing tissue extracts (peripheral wells) with lens antiserum (central well). The peripheral wells contain respectively: A, lens; B, vitreous body; C, lens; D, iris; E, aqueous humour; F, lens; G, cornea; H, retina.

(Manuscript received 26 : iii : 59)

The Organ Specific Action of Thyroxin in Visual Pigment Differentiation

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INTRODUCTION

In the course of his studies on the phylogenetic distribution of the retinal photopigments, Wald (1942, 1946, 1947) observed that the visual pigment of larvae of *Rana catesbeiana* changed from porphyropsin to rhodopsin during metamorphosis. The essential difference between the two visual pigments, which are conjugated proteins, is in the chromophore group, vitamin A aldehyde (or retinene). Vitamin A-2 aldehyde is the chromophore of porphyropsin; vitamin A-1 aldehyde, which has one less double bond in its beta ionone ring, is the chromophore of rhodopsin (reviewed by Dartnall, 1958). The phenomenon of visual pigment conversion during metamorphosis has recently been examined in detail by Wilt (1959). His findings confirmed Wald's earlier report fully; furthermore, it was demonstrated that administration of thyroxin to premetamorphic animals stimulated photopigment conversion. Other evidence was presented supporting the hypothesis that thyroxin, or its physiologically active derivative, effects a change in vitamin A metabolism which results in a change in the type of chromophore on the visual protein. This paper is a report of further attempts to determine the organ specificity of thyroxin in this system, i.e. in what organ does thyroxin exert its primary effect?

The experiments are designed to distinguish between two sites at which the hormone may effect photopigment conversion: either thyroxin acts directly on the tissues of the eye, or it acts primarily on some other organ, such as the liver or intestine, and conversion in the eye only reflects a prior metabolic change occurring elsewhere. If the first hypothesis is correct, a dose of thyroxin placed in the eye should be more effective in producing photopigment conversion than if an equivalent dose of thyroxin were placed in the abdomen. Furthermore, the eye receiving thyroxin should show a greater degree of photopigment conversion than the contralateral sham-operated eye. However, if the latter hypothesis is correct, there should be no difference between the two eyes, and thyroxin in the abdomen should be as effective or more effective than thyroxin placed in the eye.

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METHODS

Larvae of *Rana catesbeiana*, the species used in all these studies, were obtained from the Carolina Biological Supply Co., Elon College, N.C. The larvae were in their second year of development, having a total body-length of 7 to 10 cm. and hind limbs 0 to 3 mm. long. The animals were not fed and were kept at 18° to 22° C.

Thyroxin-cholesterol pellets, which were prepared as described by Kaltenbach (1953*a*), contained 20 per cent. thyroxin. Each pellet, measuring 0·5 × 0·01–0·05 mm., contained from 25 μg. to 50 μg. of thyroxin, except in one experiment in which the pellets contained 10 to 25 μg. of thyroxin per pellet. Kollros & McMurray (1956) and Kaltenbach (1953*a*) have previously shown that administration of thyroxin in this form leads to a slow release of the hormone in which diffusion is less widespread than with other vehicles.

The pellets were implanted by a simple operative procedure. After anesthetizing the larvae in MS 222, the eye was pierced at the corneal-scleral junction with an iris spear knife, and the pellet was inserted into the vitreous humour with watchmaker's forceps. The pellet invariably remained in place and was not extruded. The contralateral control eye was sham-operated, but no pellet was inserted (cholesterol alone has no effect, cf. Kaltenbach, 1953 *a*, *b*, *c*). A sharp 17-gauge syringe needle was used to pierce the abdominal wall so that a pellet could be inserted into the abdominal cavity. The animals were then placed in 50 per cent. Holtfreter's solution containing 50,000 units of penicillin and 50 mg. of streptomycin sulphate/litre and kept in the dark at 18 to 22° C. for 12 hours. In over 400 operations of this type only 3 larvae became infected as a result of the operation.

The water was subsequently changed daily, and the larvae were exposed to bright artificial light for at least 4 hours each day. After 5 to 9 days the tadpoles were anesthetized and measured, and their eyes were then removed. The cornea was cut along one side, the lens removed, and the pellet removed from experimental eyes; then the eyes were exposed to bright white light for 30 minutes. Retinene released from the visual pigment during this bleaching is quantitatively reduced *in situ* to vitamin A. The whole eyes were then ground to a dry powder with sodium sulphate and sand. This powder was extracted at 12° C. with petroleum ether in the dark for 4 to 12 hours under a nitrogen atmosphere. The petroleum ether extract was taken to dryness under reduced pressure and the residue saponified for one hour at 55° C. with 6 per cent. methanolic KOH. The methanol was diluted with water to a final concentration of 60 per cent. and the methanolic phase extracted with *n*-hexane. The *n*-hexane was washed free of base with water and then taken to dryness. The sample was finally dissolved in 0·2 to 0·4 c.c. of anhydrous chloroform.

Analysis of the two vitamins A was carried out by the sensitive antimony trichloride colorimetric reaction (cf. Wilt, 1959). The reaction is carried out in a 1·0 c.c. cuvette, the spectrum being recorded from 750 mμ to 550 mμ from 5 to 20 seconds after mixing. The DK-2 Beckman recording spectrophotometer was employed. Vitamin A-2 absorbs maximally at 695 mμ, and vitamin A-1 at 620 mμ, in this test. The proportions of the two vitamins in a mixture were calculated by use of the simultaneous equations proposed by Wald (1938). Under the condititions employed in these experiments the difference in the percentage vitamin A-1 of duplicates never exceeded 1·5 per cent.

RESULTS

Thirteen separate experiments were carried out on a total of 361 larvae. The design of each experiment was the same. An equivalent dose of 25 to 50 μg. of thyroxin was put into one eye of an animal, or into the abdominal cavity. (Only 10 to 25 μg. of thyroxin were used in series 2.) After a suitable period of time the percentage of vitamin A-1 (which is indicative of the rhodopsin content) present in eyes which received thyroxin was compared to the contralateral sham-operated eyes of the same animal, to eyes of animals receiving thyroxin in the abdomen, and to eyes of non-operated controls. Since the increase in vitamin A-1 takes place in both the retina and pigmented epithelium at the same rate (Wilt, 1959), analysis of whole eyes does not introduce any complication. Table 1 presents the results of these experiments. For each experiment the number of eyes, the duration of exposure to hormone, and a morphological index of metamorphosis (the hind limb/tail ratio) is presented. The percentage of vitamin A-1 for each group of eyes is recorded, and the difference between the percentage of vitamin A-1 present in non-operated controls and experimental groups is shown.

Intra-ocular or abdominal implantation of thyroxin-cholesterol pellets leads to a progressive increase in the percentage of vitamin A-1 in the eyes. The percentage of vitamin A-1 in non-operated control eyes is rather constant, averaging around 13·5 per cent. The variable sensitivity of different groups of animals is indicated by the different responses to the same amount of thyroxin over the same time period. For instance, in series 3, after 7 days, there is an increase of 17 per cent. vitamin A-1 in eyes which contained thyroxin, but in series 5, after 7·5 days of the same treatment, there is only an increase of 4 per cent. On the other hand, the animals in series 3 showed a change of 159 per cent. in the hind limb/tail ratio, while the comparable group in series 5 showed a change of 236 per cent. Hormone sensitivity varies in different populations of animals, and different metamorphic events show different hormone sensitivities that bear no constant relation to each other in different populations. This finding is parallel to experience with other hormone responsive systems, but the elucidation of the factors responsible for sensitivity differences in a population is still largely a matter for speculation.

The experiments do, however, provide a clear and consistent indication of the primary organ-specific action of thyroxin. In no case does the percentage of vitamin A-1 of eyes from animals with thyroxin implanted into the abdomen exceed that of the eyes from animals in which thyroxin was implanted into the eye. Rather, with one exception, the eye which had thyroxin in it consistently contains a greater proportion of vitamin A-1. In the one exceptional experiment (7-day experiment of series 2) the values are almost the same; in this case the

TABLE 1

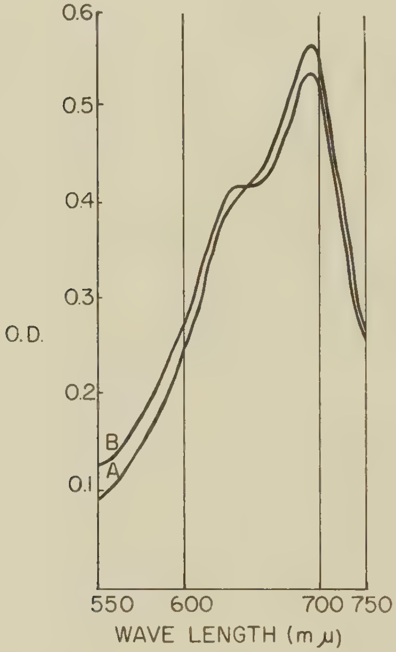
Vitamin A-1 content of thyroxin-stimulated eyes

Series	*No. of eyes*	*Duration*	*Treatment*	*Vitamin A-1*	*Increase in vitamin A-1*	*Increase in hind limb/tail ratio*
		(*days*)		(%)	(%)	(%)
1	12	8	Non-operated	16·9	. .	. .
	24		Thyroxin in eye	38·5	21·6	. .
	24		Sham-operated eye	27·0	10·1	. .
	18		Thyroxin in abdomen	31·1	14·2	. .
2*	18	5	Non-operated	13·8	. .	. .
	20		Thyroxin in eye	26·2	12·4	55
	20		Sham-operated eye	21·7	7·9	55
	20		Thyroxin in abdomen	19·5	5·7	1
2*	18	7	Non-operated	14·6	. .	. .
	23		Thyroxin in eye	28·9	14·3	88
			Sham-operated eye	26·7	12·1	88
			Thyroxin in abdomen	28·6	14·0	145
3	16	5	Non-operated	11·6	. .	. .
	19		Thyroxin in eye	14·5	2·9	52
	19		Sham-operated eye	13·7	2·1	52
3	19	6	Thyroxin in eye	22·0	10·4	170
	19		Sham-operated eye	19·0	7·5	170
3	18	7	Non-operated	10·1	. .	. .
	18		Thyroxin in eye	27·2	17·1	159
	18		Sham-operated eye	21·8	11·7	159
4 (*a*)	16	8	Non-operated	11·9	. .	. .
	17		Thyroxin in eye	18·7	6·8	87
	17		Sham-operated eye	14·4	2·5	87
	14		Thyroxin in abdomen	15·8	3·9	271
4 (*b*)	14	8	Non-operated	8·7	. .	. .
	17		Thyroxin in eye	16·2	7·5	166
	17		Sham-operated eye	12·2	3·5	166
	14		Thyroxin in abdomen	11·7	3·0	269
4 (*c*)	19	8	Thyroxin in eye	21·0	10·7	89
	19		Sham-operated eye	16·0	5·7	89
5	18	5·5	Thyroxin in eye	23·1	4·0	106
	18		Sham-operated eye	21·5	2·4	106
5	22	7·5	Non-operated	19·1	. .	. .
	15		Thyroxin in eye	23·7	4·6	236
	15		Sham-operated eye	19·5	0·4	236
	20		Thyroxin in abdomen	23·0	3·9	271
5	13	9	Thyroxin in eye	29·3	10·2	275
	13		Sham-operated eye	25·5	6·4	275
5	13	9	Thyroxin in eye	28·6	9·5	275
	13		Sham-operated eye	25·1	6·0	275

* The animals in this series were given only one-half the usual thyroxin dose.

dose of hormone was one-half of that used in the other experiments. Since there was little or no increase between 5 and 7 days in the percentage of vitamin A-1 of the eyes containing thyroxin, it is reasonable to conclude that the supply of hormone in the pellet was near depletion and any unilateral stimulation became obscured.

The consistent difference between the percentage of vitamin A-1 in eyes receiving thyroxin and eyes from animals with thyroxin in the abdomen is even more striking when it is realized that thyroxin in the abdomen is much more effective than intra-ocular thyroxin in stimulating some metamorphic events, such as hind-limb growth, tail resorption, head-shape changes, &c.



Text-fig. 1. Antimony trichloride reaction spectra for the determination of vitamins A-1 and A-2. Curve A is the reaction spectrum for an extract of eyes which had not received thyroxin. Curve B is the reaction spectrum for an extract of the contralateral eyes from the same animals. These eyes had received a thyroxin pellet 9 days prior to the determination of reaction spectra.

There is also a highly consistent and significant difference between the two eyes in animals in which only one of the two eyes received thyroxin. In most experiments, the difference is observed after 5 days of exposure to the hormone. The difference is very striking in some experiments, and it is interesting to note that the greater the effectiveness of the hormone in any experimental group, the greater the difference between eyes receiving thyroxin and the contralateral sham-operated group. If only the experiments with large hormone doses for 7 days or longer are considered, the stimulation of conversion in the eye with thyroxin is completely reproducible in 8/8 experiments; the probability that this could be a sampling error is less than 4 in 1,000. In no cases does the percentage of vitamin A-1 of the sham-operated control exceed that of eyes receiving thyroxin. Previous determinations of vitamin A-1 of pooled left and right eyes have shown that such an abnormal distribution does not exist in untreated populations (Wilt, 1959). Text-fig. 1 presents a spectrum of an antimony trichloride reaction mixture for a typical experiment. In this particular determination application of the simultaneous equations for determining the percentage of vitamin A-1 revealed a difference of 4 per cent. between the two groups of eyes. The eyes which were directly exposed to thyroxin gave a spectrum in the colorimetric test which has a flatter slope from 620 mμ to 640 mμ (the region of vitamin A-1 absorption in this test), indicating an increased concentration of vitamin A-1 in this group as compared to the sham-operated control eyes.

DISCUSSION

It is believed that the differences recorded in Table 1 are highly significant because of the reproducibility of the methods and the consistency of the results. The general picture is the appearance of a slight difference between the two eyes by 5 days after the implantation of thyroxin, the difference becoming more apparent by 7 to 9 days. These findings, and the comparison of the effect of intra-ocular implantation with abdominal implantation, clearly support the hypothesis that thyroxin acts directly on the tissues of the eye to effect in some way a change in the chromophore of the visual pigment.

The difference between sham-operated eyes and eyes with thyroxin is most marked when the increase in the percentage of vitamin A-1 is most marked, i.e. when the system is most sensitive. As mentioned before, the factors responsible for this variance in sensitivity are unknown. One difference between photopigment conversion and other metamorphic events is concerned with the methods of measurement. A doubling of the concentration of vitamin A-1 from 10 per cent. to 20 per cent. seems much less dramatic, and is certainly more difficult to measure accurately, than a doubling of hind-limb length, a process which may involve growth of a 2-mm. rudiment to a well-formed limb 4 or 5 mm. long and with well-formed digits. Second, and more important, photopigment conversion is apparently less sensitive to thyroxin than are some other metamorphic events such as mucous gland development, hind-limb growth, &c. A striking example of the difference in thresholds is a comparison between photopigment conversion and the development of the tissues surrounding the eye. Kaltenbach (1953*b*) has shown the growth of adnexa, and especially the development of eyelids and mucous glands, is apparent by 72 hours after local unilateral implantation of thyroxin-cholesterol pellets into the orbit, one or two days before the amount of ocular vitamin A-1 begins to increase noticeably. Histological examination of sections through whole larvae 3·5 days after intra-ocular implantation confirms Kaltenbach's finding; there is a slight but noticeable stimulation of mucous gland development in the orbital region of the side with thyroxin. It is also interesting to compare the general efficacy of intra-ocular and abdominal implantation. An instructive if only approximate comparison can be carried out by calculating the percentage increase of the metamorphic change per day. This is done by averaging the percentage increase of hind limb/tail ratios and the change in the percentage vitamin A-1 compared to the baseline vitamin A-1 content of non-operated controls, and then dividing these by the average number of days of exposure to thyroxin. Using this procedure the percentage increase over the baseline value/day is:

	Hind limb/tail	*Ocular vitamin A-1*
Abdominal implant	26·6	7·05
Control eye	21·9	10·6
Ocular implant		6·5

Under the specified experimental conditions the response of hind limb/tail is more rapid than the change in the percentage of vitamin A-1 of the eye, even in intra-ocular implants. While the methods of analysis are more difficult for photopigment conversion, the apparent relatively high threshold seems to be a real one, at least in an operational sense; certainly further dose/response experiments are needed. In any complex biological response in which one event has a higher threshold than others, unilateral stimulation *in vivo* will be more difficult to demonstrate clearly because of the probability of increased diffusion of the biological effector with increased time of exposure. A response which occurs in a few hours or days will show local stimulation more clearly than if the response takes much longer, because the diffusion of the agent being studied increases over a longer period of time.

The most economical hypothesis for the mechanism of thyroxin action on the biochemical level in this system is that thyroxin leads to the loss of an enzyme or enzyme-forming system concerned with vitamin A-2 synthesis. There is no direct proof for this idea, but it is interesting to notice that this hypothesis predicts an apparent low sensitivity of photopigment conversion to thyroxin. In theory, as the biosynthesis of vitamin A-2 ceases, the increase in ocular vitamin A-1 would proceed by entrance of the vitamin from surrounding tissues (only vitamin A-1 is present in other tissues of the larva; Wilt, 1959); the vitamin A-2 already present in the eye would slowly be lost by normal 'wear and tear'. In other words, there would be a slow destruction of existing molecules rather than a rapid synthesis of new enzymes and their consequent end products.

Whatever the correct interpretation, the loss of one double bond in the visual pigment chromophore during a period of intense developmental activity offers a real opportunity for analysis of differentiation on the molecular level.

SUMMARY

The stimulation of conversion of visual pigment from porphyropsin to rhodopsin in eyes of larvae of *R. catesbeiana* has been effected by local implantation of thyroxin-cholesterol pellets. An eye which has received a pellet consistently shows a greater increase in the vitamin A-1 content, an index of

rhodopsin content, than does the contralateral sham-operated eye, or eyes from animals with pellets placed in the abdominal cavity. It is concluded that photopigment conversion is a direct response of the eye to thyroxin or its physiological derivative.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the advice of Dr. James D. Ebert during the course of this work, and the generous financial support of the Carnegie Institution of Washington.

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(Manuscript received 6 : iv : 59)

Embryonic Growth relative to the Vitelline Membrane, and its Role in Determining the Level of Post-operative Abnormalities, as Measured in the Chick

by P. H. S. SILVER[1]

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In a paper concerned with the fate of the first somite in the chick embryo, Hinsch & Hamilton (1956) drew attention to the incidence of post-operative abnormalities encountered, namely, 14 out of 33 in one of their series and 22 out of 50 in another. The technique used by these workers was to make an opening in the vitelline membrane (V.M.) at the 5–6 somite stage, mark the first somite with carbon, and then allow development to proceed. The abnormalities observed included spina bifida (46·8 per cent.), 'twisted embryos', heterotaxis, and small eyes in which the lens was displaced on one or both sides of the head. They suggested that the first three abnormalities mentioned were due to the embryo pressing against the edge of the opening made in the V.M. With regard to the *level* at which spina bifida was observed they made the following interesting statement: 'the defect occurred much later and in a posterior part of the tube rather than at the level of the operation, so that trauma from the marking needle would seem an unlikely explanation'. It is with this observation that the present communication is mainly concerned. Data will be presented below to demonstrate first, the changing relationship between the first somite and the overlying V.M. as the embryo grows in length, and second, how this discrepancy between the *level* of the operation and that of the lesion can come about. Observations concerning the backward movement of Hensen's node are also included: these findings are in conformity with the well-known work of Wetzel (1929), Pasteels (1937), and Spratt (1947).

MATERIAL AND METHODS

The experiments recorded in Tables 1 and 2 were undertaken with white leghorn chick embryos. The results are based on 37 consecutive experiments. No post-operative abnormalities were seen affecting either embryo or membranes.

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In order to study the movements of the growing embryo in relation to the overlying V.M. the following very simple procedure was employed. A small puncture (0·1 mm. diam.) was made in the V.M. with a tungsten needle about 0·3 to 0·4 mm. either to the right of the 1st visible somite or to the right of Hensen's node. Minimal staining with neutral red was employed in certain cases.

Having replaced the window, the egg was topped up (Grabowski, 1956) and rolled 180° on the candler. Development was allowed to proceed for a further 24 or 48 hours, i.e. to an approximate total incubation time of 48 or 72 hours, when a new window was cut over the blastoderm. The sites of V.M. puncture were located by heavy staining with Nile blue sulphate and their relationship to the embryo recorded in terms of somite levels.

It was considered desirable at the same time to measure the distances separating the somite opposite the puncture-mark in the V.M. from the 1st somite or from Hensen's node, but in the former case, owing to the disappearance of the 1st somite, measurements were made instead to the most proximal part of the otocyst. This measurement in fact did not give an exaggerated indication of the degree to which movement had occurred owing to the flexed position of the embryo in the head region. In the case of Hensen's node no problem arose up to the time of development of the tail fold: but once the fold had developed the node was, of course, no longer visible *in ovo*. Accordingly measurements were made to the most distal visible part of the tail.

The present study was not extended beyond the end of the 3rd day since the V.M. ruptures soon afterwards. In any case by this time the embryo is enveloped in the amniotic sac and the relationship of embryo and V.M. is no longer of practical importance.

RESULTS

The data relating to the forward movement of the 1st somite relative to the V.M. are shown in Table 1 and Text-fig. 1; those relating to the backward movement of Hensen's node are shown in Table 2.

Movement of 1st somite relative to the V.M. (Table 1 and Text-fig. 1)

The first column in Table 1 shows that of 21 experiments 14 were carried out on embryos of six or seven somites, i.e. at the stage when the mid-brain folds come together; in the other seven experiments one embryo had four somites, three had eight, and three had nine.

In the second column the results have been arranged serially in each group according to the stage reached when the results were examined. If attention is directed to those embryos in which the results were examined on the day subsequent to the operation (by which time stages with from 23 to 27 somites had been reached), it will be seen that the opening made in the V.M. no longer lay opposite the 1st somite but was situated somewhere between the 10th and 14th somites (column 3). The actual distances measured from the somite opposite the opening to the otocyst varied from 1·5 mm. to 2·3 mm. (column 4). Two further cases included in the table were examined 1½ days after the experiment, and the openings were seen at the level of the 17th somite in both cases.

In the embryos examined 2 days after the operation (Hamburger & Hamilton, 1951, stages 18 or 19) the opening was situated between the 15th and 20th somites, i.e. opposite the right forelimb bud. The distances from the somite opposite the opening in the V.M. to the otocyst now ranged from 2·8 to 4·9 mm. The considerable variation in the last figures is brought about by differing degrees of head flexion, size of embryo, &c.

TABLE 1

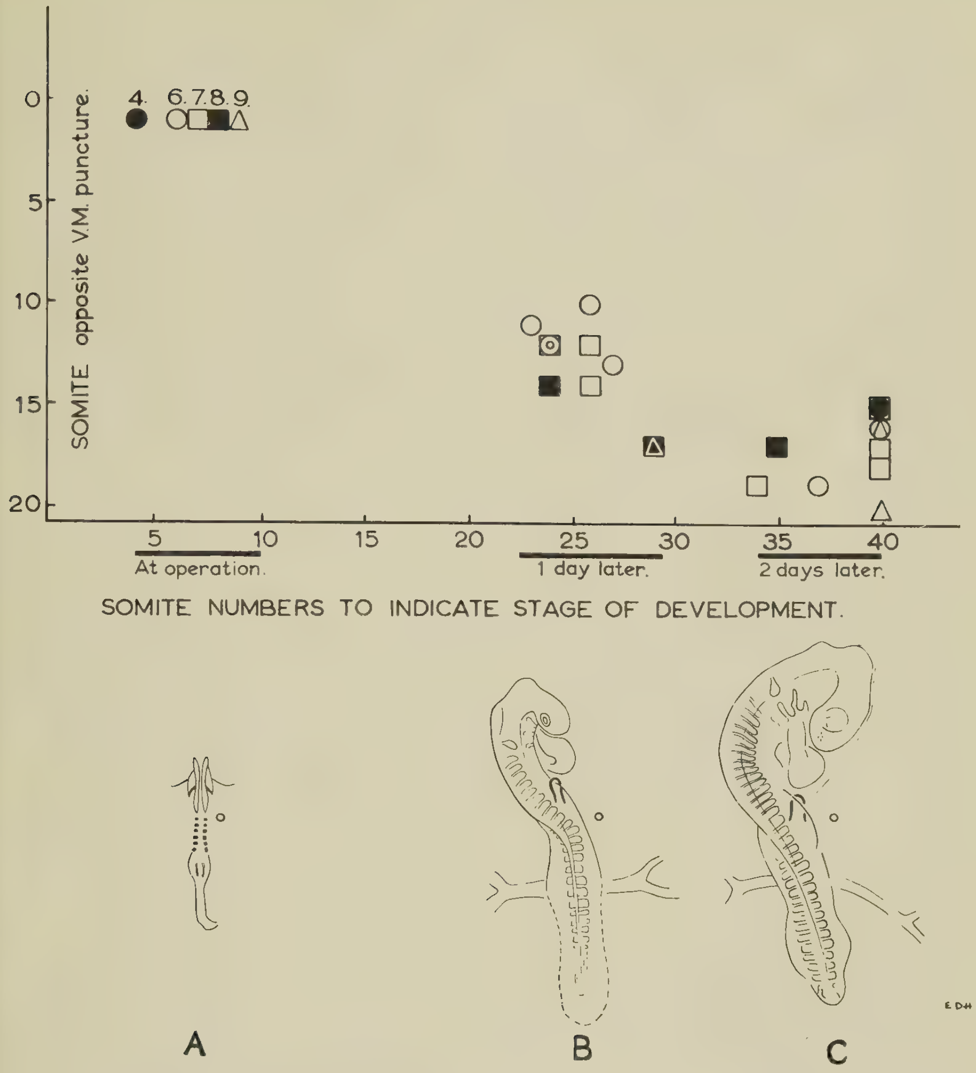
Vitelline membrane punctured opposite 1st somite

The table lists the experiments in which the V.M. was punctured opposite the 1st visible somite. The operation was carried out after *c.* 30 hours' incubation, the embryos having reached the stages of development shown in the first column. Development then proceeded for 1 or 2 days to the stages shown in the second column. Re-examination now showed the opening to be situated opposite the somite indicated in column 3, and to be separated from the otocyst (measurements not taken to the 1st somite for reasons explained in text) by the distances shown in column 4. The data contained in the first three columns are also shown graphically in Text-fig. 1

Stage at operation in somites	*Final stage of development (Hamburger and Hamilton). Somite numbers and days since operation indicated +1 or +2*	*Opening in V.M. opposite somite number*	*Distance from opening in V.M. to otocyst* (mm.)
4	stage 19, *c.* 40 somites +2	15	4·0
6	stage 14, 23 somites +1	11	1·5
6	stage 14, 24 somites +1	12	2·1
6	stage 14, 24 somites +1	12	2·3
6	stage 15, 26 somites +1	10	1·5
6	stage 15, 27 somites +1	13	1·9
6	stage 18, *c.* 37 somites +2	19	3·0
6	stage 19, *c.* 40 somites +2	16	2·8
7	stage 14, 24 somites +1	12	2·2
7	stage 15, 26 somites +1	12	1·9
7	stage 15, 26 somites +1	14	2·0
7	stage 18, 34 somites +2	19	3·2
7	stage 19, *c.* 40 somites +2	17	2·5
7	stage 19, *c.* 40 somites +2	15	4·0
7	stage 19, *c.* 40 somites +2	18	3·8
8	stage 14, 24 somites +1	14	2·0
8	stage 16, 29 somites +1½	17	1·9
8	stage 18, 35 somites +2	17	3·4
9	stage 17, 29 somites +1½	17	2·7
9	stage 19, *c.* 40 somites +2	16	4·9
9	stage 19, *c.* 40 somites +2	20	3·8

Movement of Hensen's node relative to the V.M. (Table 2)

Examining Table 2 in the same way we see that on the day subsequent to the operation (stages reached varying from 20 to 26 somites), the opening lay



TEXT-FIG. 1. This graph has been drawn using the data contained in the first three columns of Table 1, and refer to experiments in which an opening was made in the V.M. opposite the 1st somite. The stage of embryonic development (Table 1, column 2) is shown on the abscissa; the position of the opening in the V.M. relative to the embryo (Table 1, column 3) is shown on the ordinate. The stage of development in somites at the time of the initial operation (Table 1, column 1) is shown by the following symbols: ● = 4 somites; ○ = 6 somites; □ = 7 somites; ■ = 8 somites; △ = 9 somites. In certain instances, owing to the coincidence of results, certain symbols have been superimposed on each other.

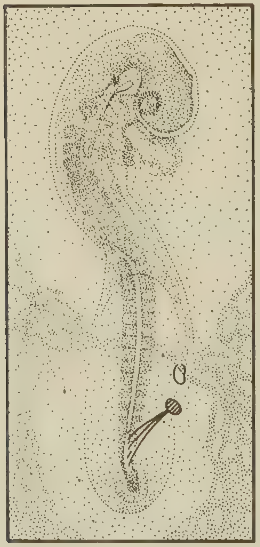
Below are shown examples visualizing stages indicated on the abscissa of the graph, A at the time of operation, B 1 day later, C 2 days later. The openings in the V.M. are shown as hollow circles. The drawings are traced from photographs taken *in ovo* and have been orientated so that the openings in the V.M. lie on the same horizon. The position of the anterior intestinal portal is indicated by a heavy curved line just caudal to the heart region. (×7 approx.)

TABLE 2

Vitelline membrane punctured opposite Hensen's node

The table lists the experiments in which the V.M. was punctured opposite Hensen's node at the stages shown in column 1. After 1 or 2 days' subsequent development (column 2) the opening in the V.M. was found opposite the somite shown in column 3, separated, from the caudal end of the embryo by the distances shown in column 4

Stage at operation in somites	*Final stage of development (Hamburger and Hamilton). Somite numbers and days since operation indicated +1 or +2*	*Opening in V.M. opposite somite number*	*Distance from Hensen's node or distal part of tail to opening in V.M.* (mm.)
3	stage 14, 23 somites +1	21	2·0
3	stage 19, *c.* 40 somites +2	19	5·0
4	stage 15, 24 somites +1	22	2·1
4	stage 19, *c.* 40 somites +2	19	4·5
5	stage 13+, 20 somites +1	10	3·1
5	stage 17, 32 somites +2	17	3·5
6	stage 14, 23 somites +1	18	2·7
6	stage 15, 26 somites +1	19	2·2
6	stage 19, *c.* 40 somites +2	26	2·8
6	stage 19, *c.* 40 somites +2	27	3·0
7	stage 15, 26 somites +1	22	1·8
7	stage 17, 31 somites +2	20	2·5
7	stage 18, 37 somites +2	26	1·9
9	stage 15, 25 somites +1	23	1·4
9	stage 19, *c.* 40 somites +2	27	3·0
9	stage 19, *c.* 40 somites +2	23	3·2



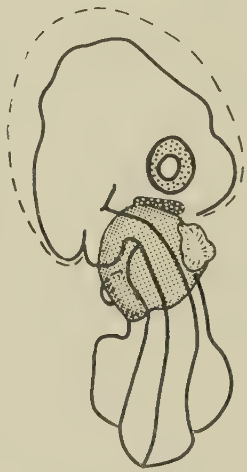
TEXT-FIG. 2. Duck embryo stage 27 somites: an opening had been made in the V.M. 0·2 mm. caudal to Hensen's node at the 14-somite stage. The hole in the V.M. is shown to the right of the 22nd and 23rd somites as an open ring. The edge of the medullary fold became adherent to the shell membrane through this opening. A long tenuous adhesion has been drawn out by the backward movement of the caudal end of the growing embryo. The tip of the adhesion (cross-hatching) flattened like a collar stud, was freed by the removal of the shell membrane and no longer lies in its original relationship to the opening in the V.M. (Drawn *in ovo.*)

between 1·8 to 3·1 mm. cranial to Hensen's node, separated from it by the whole of the undifferentiated paraxial mesoderm and the caudal 2 to 10 somites. On the second day subsequent to the operation (stages 17 to 19) the tail fold had formed and the node was no longer visible, but the opening lay between the 17th and 27th somites, a distance of between 1·9 and 5 mm. from the most caudal part of the tail fold.

The legend for Text-fig. 2 draws further attention to the relative movements of the Hensen's node region and V.M.

DISCUSSION

There is no question that adhesion to the edge of the opening in the V.M., as Hinsch & Hamilton suggest, is a cardinal cause of abnormalities in embryos operated on during the first 2 days of development. Grabowski (1956) says that this opening should not exceed 0·2 mm. diameter. The point which is brought out in the present communication is the forward movement of the cranial end of the growing embryo in relation to such an opening. If an abnormality occurs due to an adhesion, the *level* of the abnormality depends on the time which elapses between the operation and the development of the adhesion. Thus in the case we have concentrated on (the 1st somite), an immediate adhesion would produce abnormality in the hind-brain region; an adhesion occurring the next day would cause mischief in the neck, while another day later it would produce a lesion in the thoracic region, giving rise to a scoliotic or 'twisted embryo', by preventing the normal processes of rotation. Text-fig. 3 exemplifies such a lesion.



TEXT-FIG. 3. Duck embryo: an opening was made in the V.M. at the six-somite stage cranial to the developing brain. At the stage shown this opening (black stippling) has become enlarged and projecting through it is the right forelimb bud. Notice the arrested amnion (dotted line) and an adhesion to the shell membrane (white stippling). The normal rotation has not occurred behind the adhesion and the embryo shows a typical scoliotic appearance.

But Hinsch & Hamilton also refer to abnormalities in the pelvic region. Assuming that their openings in the V.M. were not excessively large, these cannot have been due to adhesion to the opening in the V.M. in their experiments but were nevertheless almost certainly due to adhesion to the intact V.M.

Naturally it will be asked how this could happen, particularly as a considerable time must have elapsed between the operation and the development of the abnormality. The following remarks may explain how this can come about. During the actual experiment, even if the whole area of exposed blastoderm is kept moist, it is impossible to avoid damaging the thin layer of albumen which normally separates the V.M. from the shell membrane. The significance of this is shown by considering the secretion of sub-blastodermal fluid (New, 1956), which takes place continuously and depends on the integrity of the layer of albumen over the V.M. from which water can be drawn. Thus, as this *supra*-blastodermal fluid is continuously being drawn upon, dehydration is continuously taking place with the threat of adhesion to the V.M. When the egg has been returned to the incubator it is significant that adhesion between the V.M. and the shell membrane (which invariably accompanies adhesion between V.M. and the blastoderm) does not occur for several hours, as is easily shown by candling; in fact it is not until much later when the embryo is completely enclosed in the amnion and the circulation is well established, that the danger of drying can be said to have passed. The earlier the stage of the embryo at operation the longer is the time which must elapse before the amnion is completed, and there is thus greater probability of abnormality in experiments at say 24 than 48 hours. This danger is specially relevant in discussing abnormalities in the pelvic region because the caudal part of the embryo is exposed for the greatest length of time.

It follows from the foregoing argument, in the view of the present author, that the solution of the problem of operating *in ovo* during the early stages, so as to avoid a high abnormality rate (see Deuchar, 1958), consists not only in providing anti-drying measures during the experiment itself but specifically in providing such measures *after* the egg has been returned to the incubator, to maintain the water content of the *supra*-blastodermal fluid. This matter will be explored in a later communication.

Lastly, when planning the site of the opening in the V.M. so as to avoid adhesion formation, it is useful to be able to picture in the mind's eye the position of the embryo a day or two after the operation when considerable growth will have occurred. It will be seen from Text-fig. 1, drawings A, B, and C, that, when development proceeds normally, the anterior intestinal portal maintains approximately its relationship to the opening in the V.M., and thus provides a region of orientation.

SUMMARY

1. The changing relationship between a point on the vitelline membrane and the 1st somite or Hensen's node has been investigated in the chick embryo, between stages of about 6 somites to about 40 somites.

2. A puncture in the V.M. opposite the 1st somite at the six-somite stage lies opposite a point between the 10th–14th somites about 24 hours later, and

between the 15th–20th somites about 48 hours later. The anterior intestinal portal maintains its orientation approximately relative to such an opening in normal development.

3. Attention is drawn to the significance of these observations regarding embryos experimented on at an early stage, and which show abnormalities far caudal to the region of operative interference.

ACKNOWLEDGEMENTS

I wish to record my thanks (1) to the Central Research Fund of the University of London for a grant for the purchase of equipment, (2) to Messrs. C. L. Jarrett and P. Runnicles, (3) to Mr. John Spurbeck for Text-fig. 2, and Miss E. D. Hewland for redrawing the other figures. I wish especially to express my indebtedness to Dr. M. E. Rawles for her guidance and encouragement over a long period, and to Professor E. W. Walls.

The subject of this paper was broached while the author was a member of the 2nd International Team at the Hubrecht Laboratorium, Utrecht. I wish to thank Dr. Pieter Nieuwkoop and his staff for their assistance at that time.

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(Manuscript received 14 : iv : 59)

The Differentiation Centre Inducing the Development from Larval to Adult Leg in *Pieris brassicae* (Lepidoptera)

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WITH TWO PLATES

IT is generally supposed that the imaginal bud of the leg in Lepidoptera, which Bodenstein (1935, 1937) reported to be visible in the second segment during the last larval instar of *Vanessa urticae*, develops to produce the adult leg by histogenesis.

Bodenstein (1935, 1937) concluded that the material in the second segment of the larval leg normally forms the anlage of the adult leg but that the regions anterior and posterior to this anlage also have the capacity to develop into a leg, though the potentiality existing in the ectoderm of such parts is not usually realized. He therefore interpreted his results from extirpation and transplantation of the legs of *Vanessa* on the hypothesis that the adult leg is derived from an imaginal bud just like the imaginal bud of a wing.

But later (1941) he wrote: 'the labile topographical anlage pattern of the leg field may be roughly as follows: the distal segments of the caterpillar leg represent the anlage of the tarsus, the second segment the anlage of the tibia and the first, the proximal segment, the anlage of the femur'. This idea served to explain the fact that duplicated or triplicated leg formation occurred in *Pyrameis* and *Phryganidia*; but it was inconsistent with his former ideas on the imaginal bud.

In fact, however, Gonin (1894) had studied the relation between the larval leg and the imaginal structure within it in *Pieris brassicae*. He termed the out-growth within the larval leg the 'femoro-tibial bud', but he did not explain how the bud developed to form the femur and tibia. He also pointed out that the trachea was bent along the epidermis of the bud, that the folds at the inside of the leg were deep, whereas those at the other side were not, and that the coxa and trochanter might be derived from the root of the leg bud. But he did not pursue the later development in any detail.

In the present paper, therefore, the post-embryonic development of the leg in *Pieris* is more fully described and some problems concerned with this development are discussed.

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MATERIAL AND METHODS

All experiments were carried out on caterpillars of *Pieris brassicae* which came from the stock culture maintained at the Entomological Field Station, University of Cambridge. The fourth, fifth, prepupal, and pupal stages of *Pieris* kept at 25° C. last 2, 3, 2, and 9 days respectively.

For histological work Carnoy's fluid was used as fixative. After fixation of the intact caterpillars for one hour the heads and abdomens were removed and the remaining parts were fixed for a further hour. Sections were cut in paraffin at 6 μ to 14 μ. Hansen's iron trioxyhaematin was mainly used for staining.

For cauterizing or transplanting the distal segments of the leg the animal was narcotized with ether. The motionless caterpillar was then placed under the binocular microscope on a mound of plasticine in which a depression was made that just about fitted the animal's body. A fine pipette, the broad end of which was connected with a rubber tube and whose narrow end exactly fitted the third segment of caterpillar's leg, was used to hold the leg during operation. This is very necessary when handling the tender larvae just after moulting. The end of the rubber tube is held in the mouth and the distal segment of the leg is sucked into the narrow end of the pipette.

This method is particularly useful when inverting the transplant; it is possible to hold the graft in position and thus to prevent the blood from flowing out. For burning restricted parts of the leg an electrically heated needle was used.

The right mesothoracic leg was used for most observations and experiments; the effects on the adult leg were observed in the pupa 2 or 3 days before emergence.

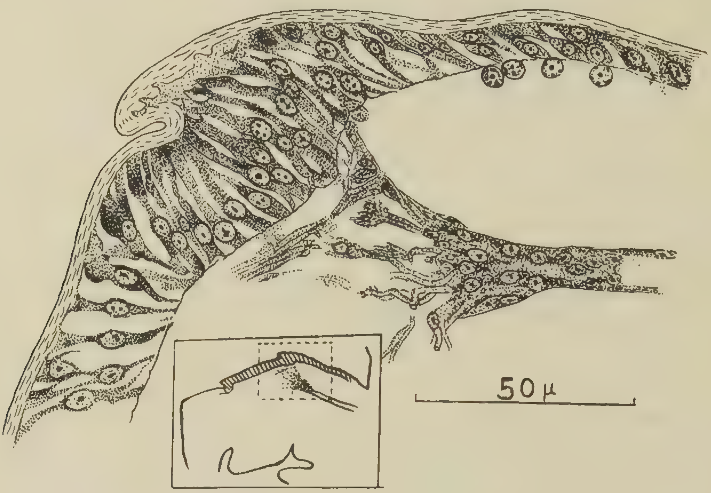
RESULTS

1. *Development of the pupal legs*

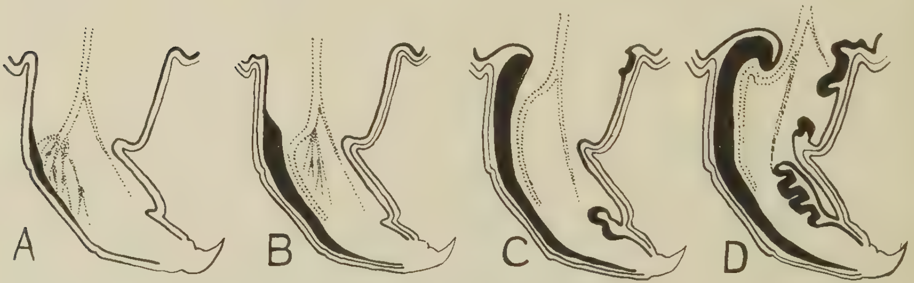
Immediately after the last larval moult the epidermis at the outer side of the third segment of the leg has elongated cells and many intercellular spaces, like the caudate cells described by Lower (1957) in the wing epidermis of Lepidoptera. The cell layer at the outer side of the second segment, on the other hand, is very thin except for a small anterior region between the second and third segments where cell divisions occur actively from the very beginning of the last instar (Text-fig. 1; Plate 1, fig. A). This region can be recognized through the surface of the soft leg after moulting because its rich tracheal supply makes it look like a pale spot. In the present paper it is regarded as a 'differentiation centre'.

The area of cellular multiplication spreads gradually from this region throughout the whole leg epidermis, especially toward the base of the second segment, to build the femoro-tibial bud. In *Pieris* the thickened part of the second segment is about 30 μ in thickness, or twice that of the general epidermis, and is conspicuous on the outer side of the second segment about 6 hours after the last moult at 25 C.; and at that time a trachea with many tracheoles is closely applied to the thickened part (Text-fig. 2).

The epidermis, which is attached to the cuticle at the beginning of the last instar, becomes separated from it about 3 hours after the last moult except at the points of attachment of the muscles. The epidermal layer can thus become thickened and folded, the thickened region of the leg agreeing closely with those parts of the leg which are sclerotized. The parts which have many bristles, that is, the inner side of the second and third segments, remain without thickening.



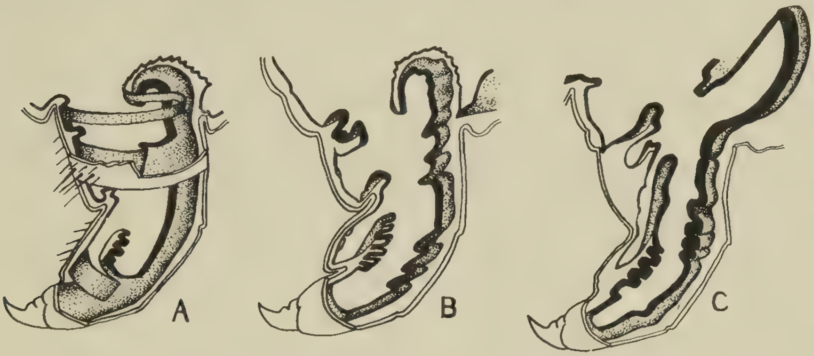
TEXT-FIG. 1. The differentiation centre in the leg, just after last moult, where cell divisions occur actively.



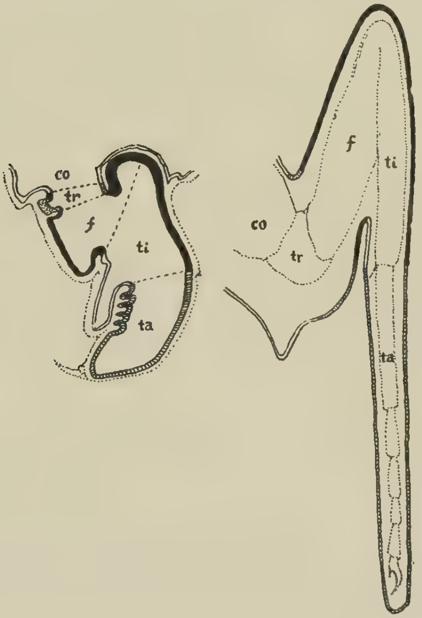
TEXT-FIG. 2. The relation between the thickened regions and the tracheae in caterpillar's legs. A, stage 3 hours after last moult. B, stage about 6 hours. C, stage at the end of the first day. D, the second-day stage.

On the second day of the fifth instar the most highly thickened part measures about 60 μ in thickness and the femoro-tibial bud begins to evaginate. The trachea, which runs immediately below the thickened epidermis, follows the evagination, as pointed out by Gonin. At this time two further thickened regions, resulting from the spread of cell divisions through the epidermis, become visible.

One is at the inner side of the articular membrane between the third and fourth segments; it is deeply invaginated, while the outside wall has been folded several times; the other is at the proximal inner side of the second segment. Later a third thickening appears between the second and third segments also.



TEXT-FIG. 3. Semi-diagrammatic drawing of post-embryonic development within the caterpillar's leg in *Pieris*. A, the second-day stage of the last instar. B, the third-day stage. C, the beginning of the prepupal stage.



TEXT-FIG. 4. Maps of presumptive areas of caterpillar's leg, and the pupal leg within which the adult leg is to be found.

Some folds which occur in the middle part of the second segment and near the top of the out-growth, as a result of active cell division, cause the evaginated out-growth to enlarge more and more. Gradually many folds appear on the third and fourth segments also and the surface area becomes very extensive (Text-fig. 3).

During the prepupal stage the epidermis retracts to form the exuvial space;

this space contains the moulting fluid, which begins to appear about 3 hours after the last moult. During this period all the folds in the epidermis extend to build the parts of the pupal leg (Plate 2, fig. F).

Ultimately the first segment builds the coxa, the thickened part at the proximal inside end of the second segment grows to form the trochanter, and the out-growth of the femoro-tibial bud forms the femur and tibia, as was pointed out by Gonin. During the prepupal period a septum appears in this out-growth, and after pupation the femur and tibia are completely separated. The third and fourth segments give rise to the tarsus. The inner part of the second segment may contribute to the side wall of the femur and that of the third segment may become the side wall of the tibia, whereas Gonin described them all as contributing to build the tarsus (Text-fig. 4).

The epidermis begins to lay down pupal cuticle on the second day after prepupation.

2. *Development of the adult legs*

At 25° C. the prepupal period lasts 2 days and then pupal ecdysis occurs. A most striking change takes place within the out-growth. Although a septum develops already during the prepupal period, it is not till the first day after pupation that pupal cuticle appears in this septum and extends from the bottom to the top of the out-growth to separate the two parts, femur and tibia (Plate 1, figs. E–H).

On the second pupal day tanning of the pupal cuticle with quinones takes place, the femur and tibia are differentiated, and then the epidermis retracts, acquiring adult characteristics progressively. The imaginal epidermis is formed at about the fourth day of pupal life and scale-forming cells are found.

In the light of these observations it is not surprising that Bodenstein (1937) obtained chimeric legs when two distal segments of the foreleg were transplanted on to corresponding part of the hind leg.

3. *Experiment on the differentiation centre*

If the second segment is removed in the third instar, as was done by Bodenstein (1933*b*), a dwarf leg regenerates (Plate 1, fig. B); if removed in the fourth or fifth instar, no leg regenerates. But when the part of the differentiation centre alone is removed or burned at the fourth instar, the corresponding adult leg has a thin tibia, especially in the proximal half, and an incomplete tarsus, though usually with the claw intact. This suggests that the regulative capacity to build the adult leg still remains at the fourth instar.

In general the new formation of the lost parts of an insect leg depends on the amount of material remaining and on the length of time available for regulation to occur. However, if the differentiation centre is removed or burned at the beginning of the fifth instar, no differentiation of the leg parts takes place in spite of the large amount of material remaining, but an out-growth resembling a larval leg with some adult characters, bearing short hairs and claw, develops at the top of the adult trochanter. The differentiation centre is clearly important for the development of the leg in its natural condition.

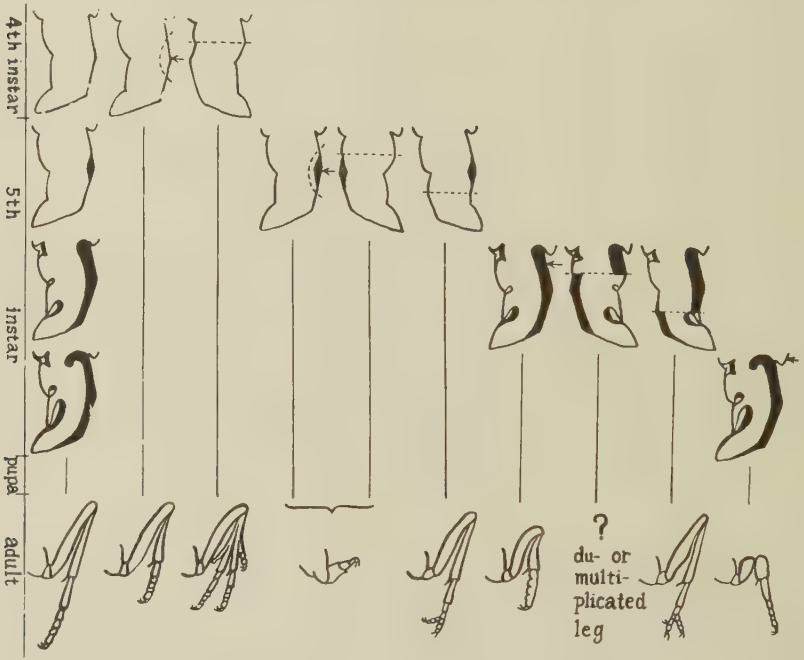
When the two and a half distal segments, including the differentiation centre, are rotated through 180° at the beginning of the last instar, to see how the thickened part appears, the distal part fails to continue its development; it degenerates, and then the larval epidermis regenerates from the stump. Finally, a larval leg-like structure develops at the top of the adult trochanter (as happens likewise after removing or burning the differentiation centre at the same stage). When the two distal segments were rotated through 180° close to the differentiation centre within the first hour after the last larval moult, and 2 days later (just before prepupation) the caterpillars were killed and sections cut, no differentiated epidermis could be found in the transplant, the epidermis of which gradually degenerated. However, when the same experiment was done on larvae about 6 hours after moulting, thickened epidermis was developing in the transplant. This was still more evident when the operation was done about 20 hours after moulting (Plate 2, figs. A–B). This result means that the thickened epidermis within the transplant, which is rotated after some degree of determination has taken place (within 12 hours after the last moult, at 25° C.), can develop (provided it has a sufficient oxygen supply, as mentioned below). But the transplant which is moved before determination gradually degenerates. After determination the thickened epidermis develops to form another leg. This result agrees entirely with Bodenstein's (1937) application of the centre hypothesis of Mangold (1929).

However, duplicated leg formation was scarcely ever obtained in *Pieris* (only two examples among 63 operated caterpillars). That is presumably because there is insufficient time available for regulation, for the last instar in *Pieris* at 25° C. lasts only 3 days, while *Phryganidia* caterpillars (Bodenstein, 1937) required 17–20 days from the time of operation to the time of pupation. Therefore, there may be another factor in addition to the differentiation centre in the production of the adult leg.

When the outer side of the first or second segment (the side on which the differentiation centre lies) is severely cauterized at some stage after the last moult, no leg develops as a rule, except the coxa and sometimes the trochanter. If the membrane between the first and second segments is cauterized in the last instar, when the femoro-tibial bud grows out conspicuously from inside the membranous region, the epidermis of the distal part, which has differentiated already, gradually degenerates (Plate 2, fig. C). This probably results from damage to the tracheae.

If, on the contrary, the outside of the second segment is burned slightly on the first day of the last instar, the adult emerging from such a caterpillar has a rather longer femur and tibia, but the tarsus is not differentiated (Plate 1, fig. C). But if the outside of the membranous segment is burned slightly on the second day, or at the beginning of the third day, the adult has a short femur and tibia and a relatively complete tarsus (Plate 1, fig. D). The burned epidermis of the second segment is considered to have approximately the same properties as the epidermis within the membranous segment which was burned later, because the femoro-tibial bud grows and evaginates. The development of an undifferentiated tarsus (Plate 1, fig. C) also depends perhaps upon the shortage of oxygen supply to the undetermined part.

When only the middle portion of the outside of the second segment is cut at the beginning of the last instar so as to damage the main trachea running into the distal part, and its inside epidermis is left without damage, an incomplete leg arises, presumably dependent on the remaining tracheoles; but an almost complete leg develops when the trachea happens to escape all damage. Naturally, in these cases, leg formation is delayed. In a single example of the last instar, sections of which were cut, the trachea had formed many new branches after wound healing (Plate 2, fig. E). On the other hand, duplicated or triplicated legs frequently develop when rotation of the distal part (comprising as many as three segments) of the limb was effected early in the fourth instar, because the damaged trachea can regenerate and often branch during the two last instars. This fact supports the suggestion that duplicated or triplicated leg formation is dependent upon development of newly formed tracheae. The experiments are summarized in Text-fig. 5.



TEXT-FIG. 5. Diagrammatic outline of experiments and their results. Arrows show cauterizations, broken lines indicate extirpations or rotations, and normal stages are given at the left of the figure.

DISCUSSION

As mentioned above, a trachea with tracheoles is found near the differentiation centre at the beginning of the last instar, and, after the cell divisions in this region, the trachea becomes closely applied to the epidermis and finally bends proximally in the second segment, following the growth of the femoro-tibial bud. The larval leg at the fourth instar has the same trachea, with some tracheoles arising from it in the same position, but no determination in the leg occurs during the fourth instar. However, some further tracheoblasts are found in this same part of the leg during the last moult (Plate 2, fig. D). These apparently lead to an increase in the number of tracheoles during the last instar, and this region, which is the differentiation centre in the natural condition, begins active cell divisions immediately after the last moult, probably because it now gets a sufficient oxygen supply. Consequently thickened regions appear successively in the different parts of the leg. It is at these stages that determination seems to take place.

According to the experimental evidence mentioned above, determination and development of the epidermis depends upon the oxygen supply. Whenever the epidermis outside the differentiation centre can obtain a sufficient oxygen supply during the early stage after the last moult, as the result of the approach of a newly formed trachea, that epidermal part likewise may be able to become a new differentiation centre. All the epidermis in the leg may well have this capacity, but those parts which are remote from the trachea in the natural condition may need some substances or stimuli for their differentiation.

The moulting hormone will induce the epidermis to develop adult characters, but it will not cause the release of the leg-forming potencies in the larval epidermis if the oxygen supply is insufficient. Stimuli of some kind from the differentiation centre, which has been activated by the moulting hormone in the presence of sufficient oxygen, seem necessary in addition and to act through the epidermis. Adult leg formation and the realization of adult characteristics in the caterpillar leg are considered to be different phenomena. The moulting hormone causes the larval epidermis of the leg to produce its imaginal characters (as in pieces of epidermis from a young larva transplanted into a later stage), but this hormone does not induce each part to develop to the corresponding part of the leg without the stimuli coming from the differentiation centre.

Since the differentiation centre may be expected to absorb more hormone than other parts, when it receives a sufficient oxygen supply from an early stage, it will differentiate at the very beginning of the last instar, and stimuli produced in it may induce cell division and self-differentiation in the remainder of the limb, provided this receives an appropriate oxygen supply. It may perhaps do this by activating the respiratory enzymes.

Williams (1951) claimed that the function of the thoracic gland hormone is to preside over the synthesis of cytochrome C, and Wigglesworth (1957) supported the view that the essential change induced by the moulting hormone is protein synthesis, and that as protein synthesis is an endothermic process for which energy is required, respiratory enzymes will be necessary. He considered two possible ways in which the moulting hormone could exert its action: the hormone might provide some material substance needed by the cells to ensure the activity of the enzymes used for protein synthesis, or act by influencing permeability within the cells, existing enzymes thus gaining access to their potential substrates. Haget (1953) has studied the gradual acquisition of the capacity for self-differentiation by the various regions of the ectoderm, and the associated loss of its ability to regulate. He has shown that the process is dependent on an influence which spreads from the differentiation centre through the sheet of ectoderm in the egg (Waddington, 1957). The observations on post-embryonic development outlined above agree with these results. Wigglesworth (1954) noted that one of the most impressive features of normal growth is synchronization and co-ordination of the process in all parts of the body. In discussing two possible media for maintaining simultaneous development, the nervous system and the epidermis, he writes that synchronous development in different parts of the integument may perhaps be maintained by chemical or other stimuli transmitted by way of the continuous epidermis. The synchronous development in the different parts of the caterpillar's leg would clearly agree with this suggestion.

Thus it would appear that in post-embryonic development the substance from the formative centre, that is, the hormone secreted by the prothoracic gland, reaches the differentiation centre of the leg which is induced to develop and to give rise to stimuli that are transmitted by way of the epidermis; just as, in the egg, embryonic development begins after chemical substances from the formative centre reach the differentiation centre. The principle of egg development, that determination proceeds step by step, agrees with that of post-embryonic development of the larval leg.

Whether the other imaginal organs have similar centres that are activated by the hormone and give rise to stimuli which induce differentiation in the surrounding parts is not known, either because they are often too small or because determination proceeds too quickly for such a centre to be recognized.

SUMMARY

1. Post-embryonic development from larval to adult leg in *Pieris brassicae* has been studied histologically.

2. It is suggested that the epidermis between the second and third segments of the caterpillar's leg, where cell divisions occur actively soon after the final moult, may be a differentiation centre which induces development from larval to adult leg, and that stimuli of some kind are transmitted from this centre by way of the continuous epidermis, producing cell divisions and determination.

3. The differentiation centre may be the region which absorbs more hormone than other parts when it receives a sufficient oxygen supply from an early stage.

4. Adult leg formation and the realization of adult characteristics in the caterpillar leg are considered to be different phenomena.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATES

PLATE 1

FIG. A. Stage about 3 hours after the last moult. *tr*, trachea; *trl*, tracheole.

FIG. B. A dwarf leg (*b*) in caterpillar which was extirpated at the third instar and then regenerated; and its section (B).

FIGS. C–D. Adult legs emerged from the caterpillar, the legs of which were cauterized slightly. C, cauterizing the outer side of the second segment of caterpillar's leg. D, cauterizing the membrane between the first and second segments at a later stage. Arrows show the cauterized areas; the legs on opposite side were left untreated as controls.

FIGS. E–H. Pupal leg just after pupation, showing division into femur and tibia. E, the upper part of the femoro-tibial bud. F, near its middle part. G, its next lower part (femur and tibia are separate). H, its lowest part. *ti*, tibia; *f*, femur.

PLATE 2

FIGS. A–B. Caterpillar's legs at the second day after rotation of the distal parts through 180°. A, rotation was made on the second day after last moult. B, rotation was made on the first day (left leg), and normal leg (right leg). Arrows show the amputated parts.

FIG. C. A degenerating larval leg arises after severely cauterizing the membrane between the first and second segments at the second-day stage. Arrow shows the area cauterized. No tracheae are visible.

FIG. D. The new epidermis in the leg during the last moult. Many tracheoblasts (*trb*) are visible.

FIG. E. Caterpillar's leg showing many tracheal branches newly formed after cutting the trachea together with the outer side of the second segment.

FIG. F. A prepupal leg newly formed in the larval leg.

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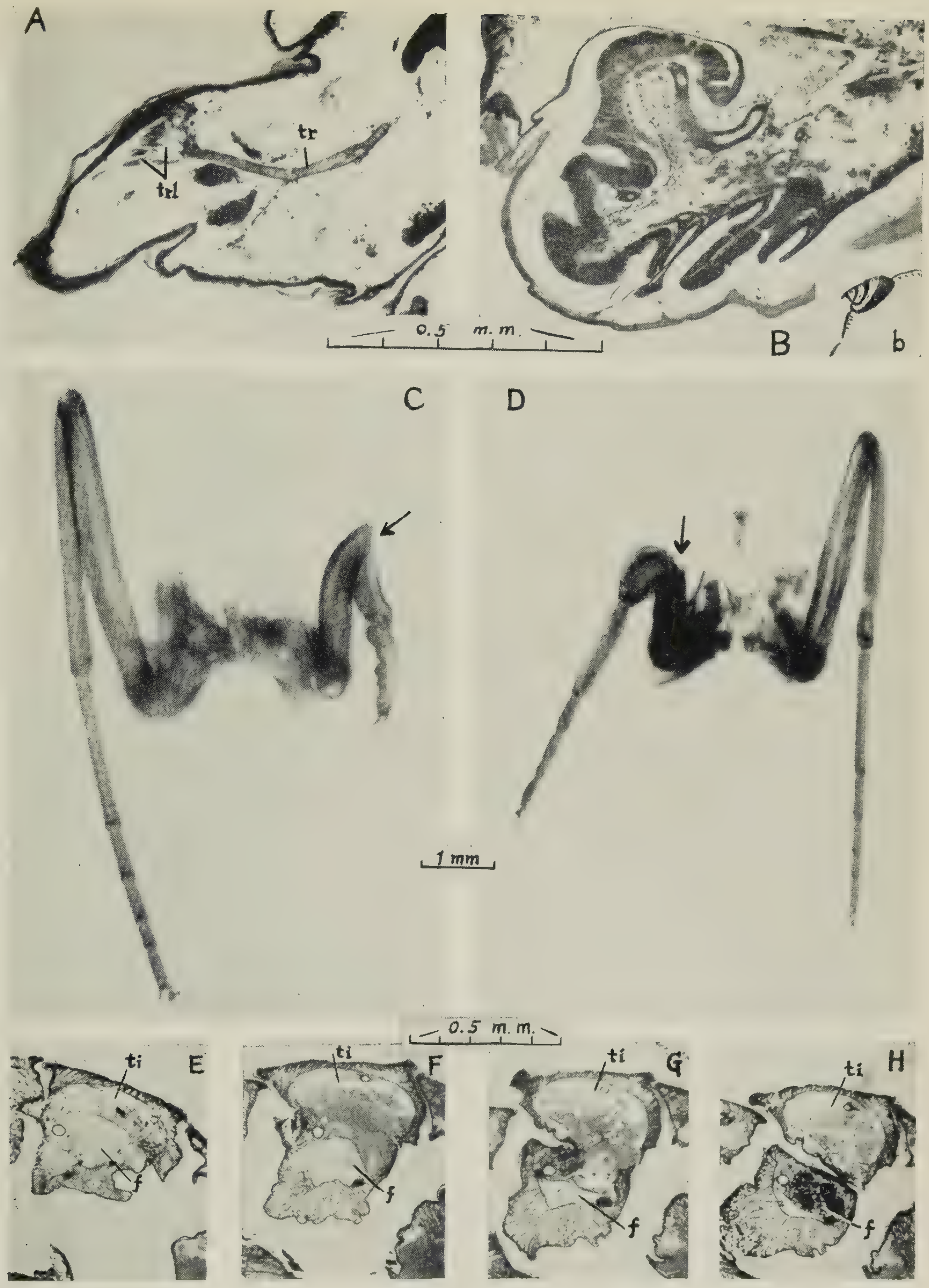
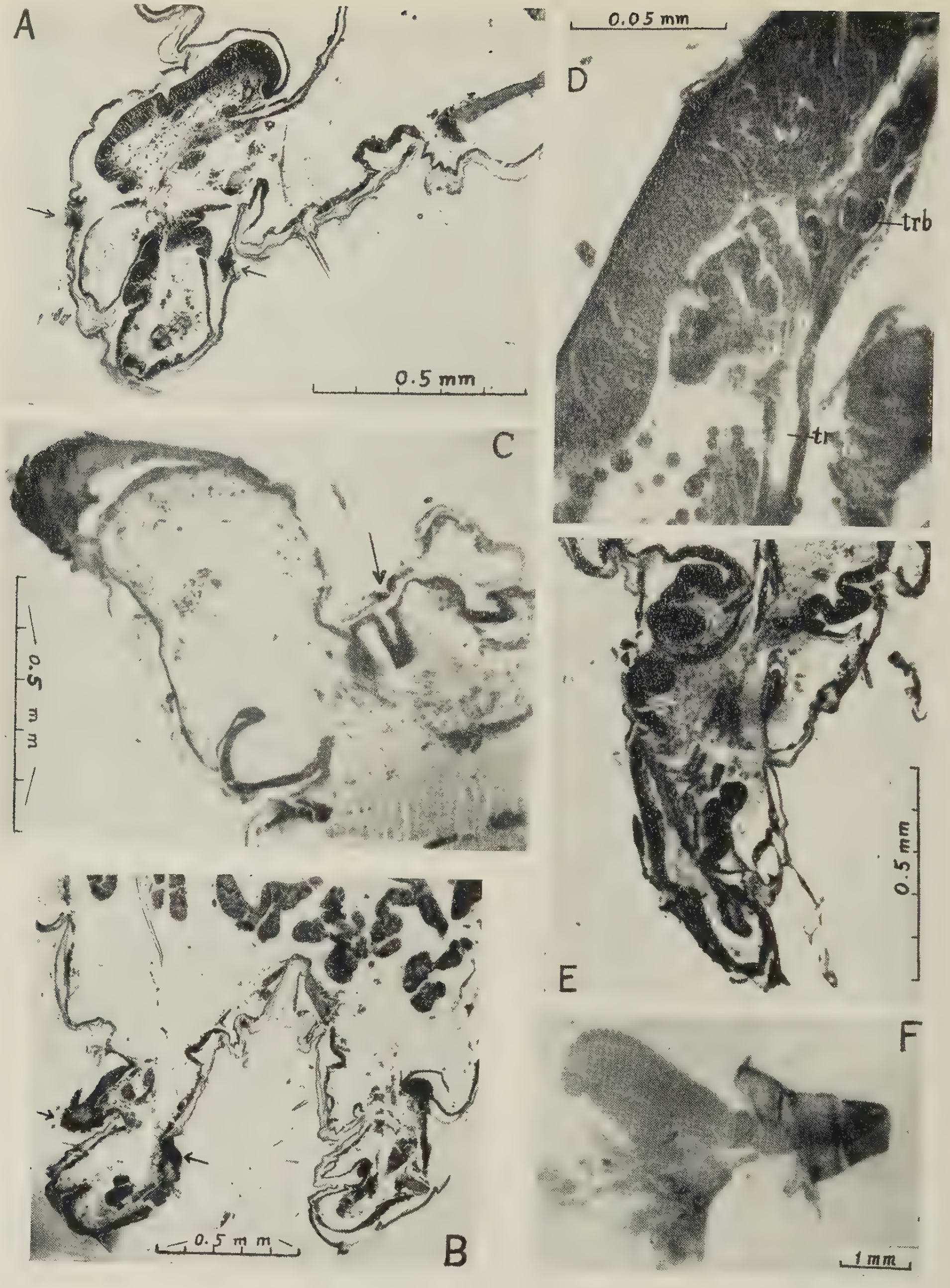


CHANG-WHAN KIM

Plate 1



CHANG-WHAN KIM

Plate 2

The Submicroscopic Structure of the *Drosophila* Egg

by EIKO OKADA[1] *and* C. H. WADDINGTON[2]
From the Institute of Animal Genetics, University of Edinburgh

WITH SEVEN PLATES

INTRODUCTION

THE wealth of mutants available in *Drosophila* provides unsurpassed opportunities for the study not only of the direct effects of genes on early embryological development but also of the complementary activities of the cytoplasm, which can be investigated in races in which 'female-sterile' genes have produced abnormal conditions in the egg. The most important pioneer in the study of gene effects was Poulson (1940), and since 1949 a number of similar problems, as well as related ones concerned with the development of female steriles, egg maturation, histo-chemistry, &c., have been taken up in this laboratory by workers such as Beatty, Yao, Counce, Ede, Pantelouris, Selman, Sirlin, Jacob (summarized Waddington, 1959). More recently a series of papers by King and his associates have discussed various aspects of the maturation of the *Drosophila* oocyte and the influence of female-sterile factors. The most recent of these papers has described some features of the submicroscopic morphology of the ovary and oocytes, particularly of the earlier stages (King & Devine, 1959). The present paper also records observations with the electron microscope; these were mostly carried out before King was kind enough to show us his then unpublished results, and they are concerned mainly with the later stages of oocyte-maturation, which he has studied in less detail.

METHODS

Drosophila melanogaster of the Oregon-K wild-type strain was used in this study. The flies were reared at 25° C. and kept for 1 or 2 days after hatching. By this time the ovaries contain many eggs at various stages of development. Etherized females were immersed in *Drosophila* culture saline (Kuroda & Tamura, 1955) and the ovaries removed and transferred to fixative without further dissection. The fixative usually employed was 1 or 2 per cent. OsO_4 buffered with veronal acetate; it was found much better to dissolve the osmic in an

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isotonic sucrose solution (Caulfield, 1957) rather than in salt solutions; the latter gave rise to an uneven clumping of the cytoplasm, which usually acquired a granular appearance and often lost all fine structure. After 1½–2 hour's fixation, the ovaries were dehydrated by passage through 30, 50, 70, 95, and 100 per cent. ethanol, transferred to a mixture of *n*-butyl and methyl methacrylate, and left to soak in a viscous methacrylate preparation for several days before being embedded at 60° C. overnight.

In ovaries fixed after the time that yolk deposition begins the opaque oocyte can easily be distinguished from the transparent nurse cell area. During dehydration the ovaries were dissected into their separate ovarioles, and each egg chamber was classified according to its developmental stage. A series of eight stages was originally set out by Yao (1949) but more recently King, Rubinson, & Smith (1957) have distinguished a further five stages during the period before oocyte growth gets under way. We have therefore used the more extensive set of numbered stages proposed by the last authors, which runs from the germarium as stage 1 to the fully mature egg at stage 14.

By stage 13 the vitelline membrane is well formed and is becoming difficult for fixatives to penetrate. From the stage of egg maturity throughout embryonic development fixation of the ooplasm, adequate for a study of submicroscopic structure, is impossible unless the membrane is pricked. Eggs were collected at known times from rapidly laying females, and after immersion in fixative were carefully pricked with a fine tungsten needle in the region farthest removed from that which it was desired to study. In some cases, after the region around the prick had coagulated, a further opening in the vitelline membrane was made, or the egg cut in half, in order to facilitate rapid penetration of the fixative. Even so the fixation of laid eggs, protected by their membrane, was never as satisfactory as that of the growing oocytes. The chorion was usually left on the eggs, since it offers little impediment to the fixative, and makes it easier to orientate the eggs during embedding. Sections were cut with a thermal advance microtome and placed on grids covered with formvar film. In some cases the sections were expanded by exposure to chloroform vapour. They were viewed with a Siemens Emiscop I. We have to thank Dr. K. Deutsch for the care of this instrument.

OBSERVATIONS

Oocyte and nurse-cell nuclei

At the beginning of oocyte growth (stage 7) the nuclei of both the oocyte and the nurse cells are smoothly rounded or spherical. However, the nuclei in these two types of cells already differ markedly in their contents. The oocyte nucleus contains a single plasmosome or nucleolus of moderate size and fairly compact shape; it frequently, perhaps always, contains a central patch of light[1] material

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[1] The words light and dark are used throughout to mean electron-transparent or electron-absorbing respectively.

(Fig. 1). (All figure numbers refer to the Plates.) The remainder of the nucleus appears considerably lighter than the cytoplasm. The membrane does not show any clear signs of doubleness, but bears a series of pores of about 650 Å diameter (Figs. 4, 19). The oocyte nucleus retains this appearance throughout the growth of the cell.

The nurse-cell nuclei at stage 7 contain much more dense material than does that of the oocyte. Much of this material is condensed into a number of rather irregular lumps, but a considerable amount of it is scattered throughout the nucleus so that the general nuclear contents is darker than in the oocyte (Fig. 2). The larger nuclear condensations often contain several patches of lighter material. In the immediately following stages (8 and 9) the quantity of dark material in the nurse-cell nuclei increases rapidly. The nucleus as a whole also enlarges, the nuclear envelope becoming thrown into a series of folds which reach an extraordinary degree of complexity by stages 10 and 11 (Figs. 6, 7). During this increase in the area of the nuclear membrane the size of the pores does not appear to change (Fig. 19). During stages 10 and 11 quite large gaps in the membrane can be found, up to 6,000 Å or even larger in size (Fig. 6). King & Devine (1959) describe similar gaps in stage 8 nuclei and present evidence that masses of material, intermediate in density between the nucleolar substance and the general nuclear contents, escape through them into the cytoplasm. At the rather later stages which we have examined most thoroughly, gaps in the nuclear membrane are often to be seen without any clearly visible 'emission body' in their neighbourhood, but in some cases the gap seems to be plugged by material which is very slightly darker than the general nuclear contents. It seems probable therefore that the emission of substance from the nucleus continues although the substance involved is not so easily recognizable. By stage 12, indeed, when the nurse-cell nuclei have reached their maximum extension and the cytoplasm has begun to shrink, the distinction between the dark nucleoli and the rest of the nucleus has become almost obliterated (Fig. 7) and the cytoplasm has also become almost as dark as the nuclear contents so that emission of substance from the nucleus would in any case be very difficult to recognize.

During stages 9 and 10 the cytoplasm in immediate contact with the nuclear envelope of the oocyte sometimes has a very fine-grained texture and appears rather less electron-dense than the main body of cytoplasm (Fig. 26). The nucleus thus appears to be surrounded by a narrow halo whose width is about twice that of the nuclear membrane. A similar appearance may sometimes be seen round the nuclei of nurse cells in these stages. Although such haloes are not found in all preparations, they occur with sufficient frequency to indicate that they have some significance.

One very remarkable nuclear inclusion has been found; it has so far only once been seen clearly in a series of sections through a stage-9 nurse cell; inspection of some photos taken earlier of other preparations has revealed two possible further examples. When the body is clearly seen (Fig. 24) it is a smooth oval profile of diameters about 2 and 3·5 μ surrounded by a well-defined membrane and containing many roughly oval dark granules about 0·1 μ in size. The membrane surrounding it is about half as thick as the nuclear envelope and does not show any sign of doubleness in this preparation; it is in immediate contact with the nuclear envelope. It is, perhaps, possible that the object is an 'emission body' of the kind described by King & Devine (1959), but their pictures do not show any bounding membrane or internal structure. At present it seems better to keep a very open mind about the nature of this nuclear inclusion, which it is hoped to study further.

Oocyte and nurse-cell cytoplasm

From stages 8 to 11 the nurse-cell cytoplasm has a rather simple appearance, consisting of a finely granular matrix in which there are embedded mitochondria, 'alpha' granules, and tubular elements (Fig. 3). The mitochondria are rather dense bodies. They vary considerably both in size and shape; usually they appear to be ellipsoidal with a minimum diameter between 0·2 and 0·5 μ and a maximum diameter perhaps 1·5 times as much, but some are considerably more elongated with lengths ranging up to as much as 2 μ. They contain quite well-arranged cristae in the form of double-membranes, which are sometimes branching, with a spacing of about 200 Å between the membranes of each pair and perhaps double that between the pairs of membranes. They are bounded by a well-marked external membrane which only occasionally shows obvious signs of doubleness. We have not seen any of the swollen mitochondria described by King & Devine (1959), which seem rather likely to be artifacts produced by their method of fixation which employed a saline fixative instead of sucrose solution.

The number of mitochondria per cell increases greatly during the growth of the nurse cells and oocyte. The mechanism of this increase is obscure. Sometimes dumbbell-shaped mitochondria are found, and these may represent stages in mitochondrial division. In certain preparations of stages 7 and 8 small mitochondria are seen with a badly developed internal cristal system but containing several very dense granules; these mitochondria are surrounded by a small space of very light cytoplasm. It is possible that they represent an early stage in the development of the mitochondria, but it appears more likely that these appearances are a consequence of inadequate fixation.

The name 'alpha granules' is used for a group of irregularly shaped dark bodies, slightly smaller than mitochondria, which are a prominent feature of the well-fixed cytoplasm of the nurse cells and oocyte in stages 7 to 11 or 12 (Figs. 3, 7). In sections they appear roughly star-shaped or as elongated strands with strongly scalloped edges. No internal structure is discernible. These granules are probably composed of lipids; and in badly fixed material several other types of inclusions appear which almost certainly represent the same cell-organelle. In some preparations, for instance, typical alpha granules are absent but there are many holes in the section; these cavities may sometimes contain a central dark irregularly shaped granule or a vague network of material of moderate density or they may be completely empty (Figs. 9, 14, 17, 22). In oocytes in which the vitelline membrane is well developed and fixation therefore somewhat slow, we have rarely seen good alpha granules, and these various types of relatively empty spaces seem to take their place.

The cytoplasm also contains a number of elongated double structures. They may be up to at least 2 μ in length, and in section consist of two membranes separated by a space of about 300 Å; the outer sides of the membranes bear a series of dense granules of about 20 to 30 Å diameter (Figs. 3, 14, 17). Serial sections show that these 'membranes' are actually sections through tubular elements, and cross-sections of them can be found in the cytoplasm although these are not nearly so striking as sections which lie along the length of the tubes. The tubes are often branched, and it is fairly frequent to find that one end is associated with a mitochondrion or alpha-granule, although there are also many such elements in which no such association can be seen.

By stages 10 and 11 the nurse cells, which had been tightly packed together, start to become rounded off. The resulting intercellular spaces are at first filled with strongly absorbing substance. By stage 12 the nurse cells have shrunk to a great extent, the nuclear membranes have reached the maximum degree of undulation, and the nuclei consist of an agglomeration of the extremely undulated membranes, dark granulated nucleolar material, and dense granules. There has been a relatively greater shrinkage of cytoplasmic than of nuclear material, the nurse-cell membrane lying fairly close to the nuclear mass. The intercellular spaces are much larger than in earlier stages and are filled with spherical bodies of various sizes, some of which consist of small vacuoles and granules. The ground substance of these spaces can be distinguished from that of the nurse-cell cytoplasm in that it is coarser and more granular, and running through it are single membranes, which seem to be extensions of the cell membranes, connecting one cell to the next. The degeneration continues until all that remains is a small amount of debris lying between the oocyte and the follicle cells in the anterior region (cf. Waddington & Okada, 1959).

During the latter part of this degeneration, for instance in stage 13, well-developed ergastoplasmic lamellar structures appear in the nurse-cell and follicle-cell cytoplasm. These may take the form of parallel double-sheets or of similar sheets arranged concentrically like the skins of an onion (Fig. 11). Such structures are much more fully developed in the degenerating cytoplasm than in the growing nurse cells or oocytes. In the nearly mature oocyte (stage 13) some rather poorly developed systems of concentric lamellae have been seen (Fig. 15). They usually enclose central regions of normal or slightly dense ground substance. It is claimed (King & Devine, 1959; Sirlin & Jacob, 1959) that during oocyte growth gaps appear in the cell membrane separating the oocyte from the nurse cells, and that nurse-cell cytoplasm moves bodily into the oocyte. If this is so the concentric formations seen in the oocyte may have been transferred in this way from the nurse cells and are in process of reorganization into ooplasmic constituents (probably mainly into ground substance, though possibly into the beta or gamma granules described below). However, the reality of these gaps in the cell membranes is not fully established (see below).

The bulk of the oocyte cytoplasm has the same appearance as that of the nurse cells; it consists of a finely granular ground substance containing mitochondria, alpha-granules, and tubular elements. The periplasm retains essentially this constitution until the egg is mature; there is, however, a definite differentiation of the cortex, which will be discussed below in connexion with the formation of the vitelline membrane; and one new cytoplasmic element, the 'lamellar stack' begins to appear at about stage 8, while another, the beta-granules, are formed in stage 13. In the more central parts of the oocyte, yolk granules form from stage 8 onwards and gamma granules can be detected in later stages.

The lamellar stacks consist of sets of parallel structures, usually some 20–30 in number, which in section appear as double membranes (Figs. 17, 18). The membranes are considerably thicker and somewhat less dark than those enclosing the tubular elements, and serial sections show that in the stacks one is dealing with extended lamellae and not with tubes. The space between lamellae is about 0·1 μ. The edges of neighbouring lamellae in some cases, but not in all, appear to bend round and join up with one another; it is also common to find a tubular element attached to the edge of a lamella. In tangential sections the lamellae exhibit a series of pores, which greatly resemble those of the nuclear envelope (Fig. 18). Since small lamellar stacks may sometimes be found in the immediate neighbourhood of the nuclear membrane, it is tempting to suggest that this membrane plays a part in their formation (cf. Swift, 1956), but many lamellar stacks are found at considerable distances from any nucleus, and this mode of origin for them remains uncertain.

In the peripheral cytoplasm of the late oocyte (stage 13), a prominent feature is the presence of large well-defined areas of light density (Figs. 14, 22). They are of approximately the same size as yolk granules or rather larger, say 3 to 5 μ in diameter. They are referred to as beta granules. Their origin is obscure, but it seems possible that they are derived by a transformation of the nurse-cell cytoplasm which is injected into the oocyte at about this time. From the time of their first formation they are found also in the deeper parts of the egg, and in early embryonic development they tend to accumulate there.

Yolk granules begin to appear about stage 7 to 8. They are usually in the form of dark slightly oval bodies, about 1 to 3 μ in diameter. In the early stages there is no well-defined limiting membrane surrounding them, and they usually exhibit very little internal structure. In some preparations, however, they can be seen to be made up of smaller particles, about 0·2 to 0·4 μ in size. One such particle is seen in Fig. 17; in other granules up to 20 of them have been seen. Structureless particles of this size may be seen isolated in the cytoplasm, and these might be either small mitochondria or yolk-granule precursors which have not yet joined up into the typical larger masses.

Scattered amongst the yolk granules there are some bodies of similar or rather smaller size, which have ill-defined though roughly oval outlines and an internal structure of fine granulations. These are referred to as gamma granules (Figs. 15, 22). They are typically considerably darker and smaller than the beta granules but, particularly in the late oocyte and early embryo (Fig. 20), there are intergrades between these two types of inclusion, and it seems not unlikely that they are two appearances of essentially the same structures.

The cortical differentiation of the oocyte, vitelline membrane formation, and the follicle cells

After the oocyte begins to enlarge, the greater part of its surface lies against the follicle cells. In stage 6 these cells become columnar over the oocyte, although remaining epithelial in the region where they abut on the nurse cells. In the follicle cells the nuclear envelope clearly shows the double-membrane appearance which is typical of most tissue-cells (Fig. 5), and therefore differs markedly from the nurse cells or oocyte nuclear envelope, in which the doubleness, if present, is not easily visible, but where one sees well-developed pores.

The cell membrane between the oocyte and the follicle cells begins to become folded in stage 7, and at that time is already rather denser than the membranes between the follicle cells. During stage 8 the foldings of the follicle cell-oocyte membranes become more elaborate (Fig. 13), and towards the end of this stage a dense material begins to be secreted within the folds. This material tends to be grouped into a series of irregular 'vitelline bodies' (Figs. 9, 23, 25). The ooplasm immediately beneath them is full of small vacuoles, which probably represent the process of secretion. During stages 9 and 10 the vitelline bodies increase in size and fuse together to form a continuous layer, which at first has a mesh-like structure (Fig. 8). At this stage the chorion begins to be secreted by the follicle cells, which themselves degenerate, showing, as they do so, well-developed ergastoplasmic structures outside the developing vitelline membrane. In the later stages of oocyte development the latter becomes thinner and more compact, losing its mesh-like appearance (Fig. 16). As is well known, the fully formed vitelline membrane is extremely impermeable.

At a time when the vitelline bodies are already formed between the oocyte and follicle cells, the membrane between the oocyte and nurse cells still remains quite thin (Fig. 9). Light microscopical and autoradiographic evidence (King & Devine, 1959; Sirlin & Jacob, 1959) strongly suggests that at certain times this membrane breaks down, at least partially, so that nurse-cell cytoplasm moves in bulk into the oocyte. We have not seen such a broken membrane in our electron microscope preparations, but King & Devine (1959) have illustrated what they claim to be such a pore at stage 8. In our sections of similar and later stages, the nurse-cell-oocyte membrane is complete. It seems likely therefore that this membrane can be broken and then remade. It is noteworthy that it often has a rather straight course and simple structure for considerable distances, but at certain places shows extreme folding and complication (Fig. 10). These regions may in the early stages (7 and 8) be connected with the appearance and disappearance of the intercellular membrane. At later stages (9, 10) they are almost certainly involved in the secretion of the vitelline membrane which appears between oocyte and nurse cells with a structure identical with that found between oocyte and follicle cells. (See also Waddington & Okada, 1959).

Beneath the developing vitelline membrane the cortical region of ooplasm is at first filled with small hollow vesicles. This condition persists until (stage 12) the vitelline membrane attains its full thickness (with mesh-like structure). From that time onwards, while the membrane is contracting and becoming more condensed, the underlying cortical ooplasm develops a surface structure which becomes thrown into a series of deep folds which extend approximately 1 μ into the egg (Figs. 8, 14, 16). The folds appear usually to be double and have dimensions rather similar to those of the cytoplasmic tubular elements. At the internal margin of the folds there are a number of dark granules about 0·1 μ in diameter ('delta' granules).

Early stages of embryogenesis

We have as yet no sections which show the maturation divisions of the oocyte nucleus or fertilization. Cleavage nuclei have been seen embedded in the internal cytoplasm before reaching the peripheral cytoplasm. They have nuclear envelopes of the usual kind with well-defined double membranes, and internally show a moderate density with no sign of any nucleolus (though it is not impossible that a small condensation of material may be present but not cut in the sections available to us). The nuclei retain essentially the same condition while moving to the surface and forming the syncytial blastoderm (Fig. 21). By the time cell boundaries begin to appear between them, a small irregularly shaped condensed area (or nucleolus) has begun to appear.

The main change which occurs in the bulk of the oocyte during this period is an increasing segregation into a peripheral region containing mitochondria, tubular elements, and alpha granules (Fig. 14), and a central portion in which the yolk and beta and gamma granules become concentrated. The yolk granules begin to be surrounded by haloes which are either quite clear or contain a loose cloud of material (Fig. 20). Presumably these are produced by the digestion of the yolk. In some cases beta granules protrude into the haloes, and the beta granules also seem to fuse with one another; one may suppose that they have a fluid consistency. There is a considerable range in electron density in these granules, and a distinction between beta and gamma granules is difficult to draw.

Eggs at this stage have to be pricked for fixation, and their surface usually withdraws somewhat from the vitelline membrane as a result of the outflow of material through the prick. In consequence of this, the cortical layer usually presents the appearance of a series of small lobular protrusions with dimensions of a few tenths of a micron (Fig. 12). Where the cortex remains firmly against the vitelline membrane one can see that it still has the same folded or spongy structure as in the mature oocyte, but at this stage the small delta granules at the base of the folds are not visible. The lobulations, when they occur, are surprisingly dark, but contain no internal structure other than minute granulations similar to the ground substance of the cytoplasm.

In the posterior region, and so far not in the anterior, of the cleaving egg a new type of granule (the 'epsilon' granule) has been seen. At a slightly later stage they can be found in some quantity in the pole cells (Fig. 21), and they probably represent the 'pole-cell granules' described from light microscopical studies, but their identification with these requires further study, which is now in progress. They are somewhat larger than the mitochondria, roughly ovoid in shape, bounded with a definite membrane which shows little sign of doubleness, and have a rather vacuolated internal structure. They have not yet been seen in the mature oocyte, but we cannot exclude their possible existence at that stage.

As the syncytial blastoderm forms, the cortex begins to lose its tendency to form protrusions, even when it has contracted away from the vitelline membrane, and by the time cell boundaries appear between the nuclei of the blastoderm, the egg surface remains smooth or gently waved after fixation. The intercellular membranes are at first extremely fine, being considerably thinner than the nuclear membranes. The formation of the cellular blastoderm, and its subsequent history, will be considered in a later paper.

DISCUSSION

This paper is intended to provide only a preliminary inventory of the constituents of the *Drosophila* egg, and it would be premature to attempt a full-scale discussion of the role which these various elements may play in development. It is hoped that such an evaluation will gradually become possible as data accumulate from two further investigations which are already in progress, firstly on the correlation between histochemical and ultra-structural appearances, and, secondly, on the effects of female-sterility genes.

Although we are obviously only at the very beginning of our knowledge about ultra-structure in eggs and early embryonic development throughout the animal kingdom, some points of interest already emerge from a comparison between the *Drosophila* egg and those few others which have as yet been studied. We find in the *Drosophila* ooplasm a rather rich variety of organelles, comprising several different types of granules as well as lamellar and tubular elements. Further, several of these types of organelles exhibit quite complex internal structure. The mitochondria of the *Drosophila* egg, for instance, have a well-developed system of cristae. In some other types of eggs, such as those of the Amphibia, the number of different kinds of organelle seems to be more restricted, and the mitochondria have a considerably simpler structure than is found in adult tissues (Eakin

& Lehmann, 1957; Karasaki, 1959; unpublished observations of this laboratory). Two factors immediately come to mind as possibly relevant to this comparison. In the first place, the maturation of the *Drosophila* egg involves the wholesale transfer into the oocyte of masses of nurse-cell cytoplasm. Although some of this cytoplasm certainly becomes broken down into the 'intercellular debris' lying between the degenerating nurse cells, and thus loses much of its initially elaborate structure before moving into the oocyte, another portion of it seems to be transferred without any breakdown through gaps appearing in the membranes between the nurse cells and oocyte. The well-developed mitochondria of the egg may therefore have arisen within the nurse cells. In the second place, it might be that eggs which exhibit a high degree of determination at the time of laying ('mosaic eggs') have better-developed internal organelles than do the more labile 'regulation' eggs such as those of Amphibia. We require data on a much wider range of material than is available at present to determine whether this suggestion is valid.

A number of interesting problems concerning the nuclear envelope are raised by the observations recorded above. In the first place, it is worthy of note that this structure has quite different appearances in the follicle cells and in the nurse cells. In the former it is clearly double; while in the latter the doubleness, if present, is not at all obvious, but the membrane exhibits very clear annuli or pores. It may well be that these apparent differences are to some extent deceptive, and that the nuclear envelopes of both types of cells are based on a constant essential basic pattern, various elements of which may be more or less strongly developed in cells of different kinds. The fact that a structure based on a double membrane, which bears annular pores, has been found in such different cells as newt oocytes (Callan & Tomlin, 1950), echinoderm eggs (Afzelius, 1955), amoebae (Pappas, 1956), to name only a few, strongly suggests that there is some basic pattern for this organelle. In amoeba the basic pattern is complicated by the development of an associated structure of hexagonal tubes. The facts described here make it clear that the possibility of considerable variation from tissue to tissue within the same organism should also not be overlooked.

During the growth of the nurse cell there is certainly a very active protein and nucleic acid metabolism (cf. Sirlin & Jacob, 1959). In connexion with another example of rapid protein synthesis in *Drosophila*, that occurring in the salivary gland cells towards the end of larval life, Gay (1956) has described the appearance of numerous 'blebs', or small protrusions of the nuclear membrane, which are thought to play a part in the transfer of material from the nucleus into the cytoplasm. In the nurse cells we have not seen such blebs. On the other hand, two other phenomena occur which seem to be connected with the transmission of nuclear influences to the cytoplasm. The first is the appearance of quite large gaps or holes in the nuclear envelope; material would be almost certain to pass through such holes, and King & Devine (1959) claim actually to have detected the passage of material from nucleus to cytoplasm in this way. Secondly, there is a very great growth in area of the nuclear membrane, which must certainly facilitate its action on the cytoplasm. The mechanism of the areal growth presents some interesting questions; it does not appear that the size of the individual pores or annuli increases, so there must be a multiplication of them, and the method by which this comes about is as yet quite unclear. Again we are struck by the differences in the structure of the nuclear membrane in two tissues of the same species—nurse cells and salivary gland cells—both of which are engaged in broadly similar activities of rapid synthesis.

Among the cytoplasmic structures, the stacks of annulated lamellae are perhaps the most striking. Similar organelles have been described in a variety of cells from various groups and their resemblance to the nuclear membrane pointed out. There has been some discussion as to whether such lamellae are to be regarded as a specialized type of ergastoplasm, and / or whether they originate directly from the nuclear membrane (cf. Afzelius, 1955, 1957; Pasteels, Castiaux, & Vandermeerssche, 1959; Rebhun, 1956; Swift, 1956); if they do form from the nuclear envelope, they might be produced by the envelope while it remains in position around the nucleus, or they might represent fragments of membrane resulting from the breakdown of the nucleus at the time of the reduction divisions. The occurrence of several such membranes arranged parallel to one another has been attributed by Afzelius (1957) to some attractive force which tends to cause isolated fragments to move into such a relationship. The frequency with which quite numerous and well-organized stacks may be seen, both in *Drosophila* and in other species (Swift, 1956) makes this explanation appear unlikely, and suggests that synthetic processes occur in the cytoplasm by which the number of lamellae becomes increased. For the initiation of such stacks there is, in *Drosophila* oocytes, a rather unusual source which might be considered. The material passed into the oocytes from the nurse cells may well contain fragments of the extensive nuclear envelopes of the nurse cells, and these might act as foci at which synthesis of further annulated lamina material might occur. Since the stacks are often found at considerable distances from the oocyte nucleus, it certainly seems unlikely that they are initiated only in its immediate vicinity, although this possibility cannot be excluded.

Ergastoplasmic structures are in general rather poorly developed in the ooplasm, being represented in the sections mainly by sinuous double-contours, which in some cases certainly, and in others most probably, represent tubular elements. The comparatively rare examples of parallel membraneous structures (other than lamellar stacks) usually represent concentric oval sheets, and may well be derived rather directly from the nurse-cell cytoplasm. The formation of elaborate systems of membranes, often arranged in a rather orderly fashion, is a somewhat unexpected feature of these cells at a time when they are clearly in process of degeneration. Similar structures are still more striking in the degenerating ovaries of some female-sterile mutants, which will be described in a later paper.

The elaborately folded cortical plasm has, perhaps, some general resemblance to the cortex of the frog oocyte, in which Kemp (1956) has described a layer of 'micro-villi'. In *Drosophila* it is not quite clear whether the contours seen in the sections represent folds or more or less longitudinal sections along minute tubules similar to those described by Kemp, although the former seems more probable. It may be noted, however, that the cortical zone in *Drosophila* is separated from the follicle cells by the thick vitelline membrane, and no question arises of any interdigitation of cortical micro-villi by follicle processes, as this author has suggested in the frog.

SUMMARY

1. A study has been made with the electron microscope of the structure of the growing oocyte and associated nurse and follicle cells in *Drosophila melanogaster*.

2. The fully grown oocyte has a cortical plasm which is thrown into deep folds, at the base of which are small delta granules. Immediately below this is a zone of periplasm which contains: well-developed mitochondria; some ergastoplasmic elements, probably mainly tubular, though sometimes taking the form of concentric oval sheets; many alpha granules which when well fixed are irregularly star-shaped and are probably lipoidal in composition; a number of stacks of annulate lamellae. Yolk granules, which may be built of a few tens of smaller particles, are found mainly at deeper levels, and so are the electron-light and probably fluid beta granules and the rather darker gamma granules. Epsilon granules, with a definite membrane and vacuolated interior, occur mainly in the posterior end of the egg and later in the pole cells.

3. The vitelline membrane, which in earlier stages has a spongy structure, begins to appear between oocyte and follicle cells earlier than below the nurse cells. The appearance of small vesicles in the ooplasm beneath the developing membrane suggests that it is secreted mainly by the oocyte. There is very complex folding of the membranes between oocyte and follicle cells and still more between oocyte and nurse cells.

4. Attention is drawn to: (i) the great enlargement in the area of the nurse-cell nuclear membranes, (ii) the striking differences in appearance between the nuclear membranes in the nurse cells and follicle cells, (iii) the formation of elaborately organized ergastoplasmic structures in the cytoplasm of the degenerating nurse cells, (iv) the large number and orderly arrangement of the elements in the stacks of annulate lamellae, which is held to suggest a process of *in situ* synthesis.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Nucleus of oocyte, stage 7. Smooth oval outline, one nucleolus containing a single lighter inclusion. Parts of two follicle cells are visible at top left.

FIG. 2. Part of nurse-cell nucleus, stage 11. The section shows one of several large nucleolar masses, each containing several lighter inclusions. Considerable amounts of nucleolus-like material are scattered throughout the nucleoplasm. The dense cytoplasm contains some mitochondria and tubular or lamellar elements.

FIG. 3. Nurse-cell cytoplasm, stage 10. Mitochondria (*Mit.*), alpha granules (α), tubular elements (*t.e.*) to the outer side of which micro-granules are attached; there are also many of these micro-granules in the ground cytoplasm.

FIG. 4. Membrane between nucleus (*N*) and cytoplasm (*C*) of nurse cell, stage 10. The section is about perpendicular to the membrane; note the appearance of cup-shaped cavities, some of which face inwards, some outwards.

FIG. 5. Membrane between nucleus (*N*) and cytoplasm (*C*) of a follicle cell, stage 7–8. A double membrane without well-developed cup-shaped cavities; characteristic ergastoplasmic structures in cytoplasm.

PLATE 2

FIG. 6. Nurse-cell nuclear membrane, stage 10, showing a typical gap. Note double membrane (?) or double rod-like structures at arrows, both in the cytoplasm (*C*), and in the nucleus (*N*), where they lie near a nucleolar mass. At *P*, an apparent pore through the membrane; at *O* a cup-shaped cavity opening outwards, at *I* a similar cavity opening inwards.

FIG. 7. Part of a section through a nurse cell, stage 11, to show the highly folded nuclear membrane. *N*, nucleus; *C*, cytoplasm.

FIG. 8. Outermost layers of oocyte, stage 12. *Ch*, inner layer of chorion, secreted by follicle cells; *V.M.* vitelline membrane which still has a spongy structure; below this is the cortical plasm of the oocyte, the plasma membrane being thrown into complex folds; in the deeper regions of these folds lie delta granules (δ).

PLATE 3

FIG. 9. Part of an oocyte (*OO.*) at stage 8–9, lying between follicle cells (*F.C.*) and nurse cells (*N.C.*). Vitelline bodies (*V.B.*) are forming between the oocyte follicle cells but not yet between it and the nurse cells. *Y*, yolk granules.

FIG. 10. Part of the membrane between nurse cells (*N.C.*) and oocyte (*OO.*); a mitochondrion (*Mit.*) is visible lower right. Stage 8.

FIG. 11. Cytoplasm in a degenerating follicle cell, stage 13, showing mitochondria (*Mit.*) and an elaborate series of concentric double lamellae.

FIG. 12. Surface of egg, 30 ± 10 min. after laying. Beneath the chorion (*Ch.*) and vitelline membrane (*V.M.*), the cortical zone forms small lobulations, probably as a result of the decreased internal pressure resulting from the prick necessary to allow penetration of the fixative.

FIG. 13. Membrane between oocyte (*OO.*) and follicle cells (*F.C.*), stage 8, just before the deposition of the vitelline membrane begins.

PLATE 4

FIG. 14. Oocyte, stage 14. Under the vitelline membrane (*V.M.*) is the folded cortical plasm (*C.P.*); the deeper regions contain mitochondria (*Mit.*), yolk granules (*Y*), alpha (α) and beta (β) granules, tubular or membraneous elements (*t.e.?*); at *A.L.S.* an annulated laminar stack.

FIG. 15. Ooocyte cytoplasm, stage 13, with mitochondria (*Mit.*), yolk (*Y*) and gamma (γ) granules, and a concentric laminar system (*C.L.S.*) enclosing material of medium density.

FIG. 16. Folded cortical membrane of oocyte, stage 13–14, beneath the vitelline membrane.

PLATE 5

FIG. 17. Posterior ooplasm, stage 13–14. The yolk granule (*Y*) shows one internal darker granule. Near it is an annulated laminar stack (*A.L.S.*); some of the laminae are connected with tubular elements (*t.e.*). The cavities in the slide probably represent badly fixed alpha granules (α?).

FIG. 18. Ooplasm, stage 13. The annulated laminar stack (*A.L.S.*) is cut nearly tangentially to the laminae so that the annulae are clearly seen.

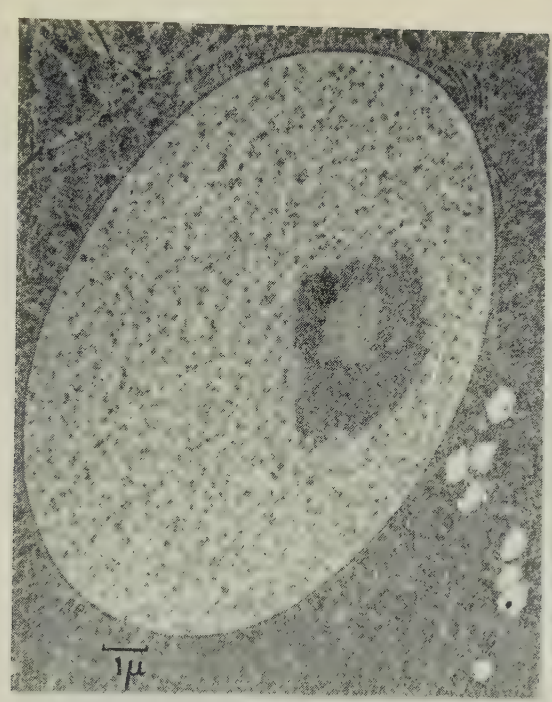
FIG. 19. Higher magnification of part of a nurse-cell nuclear membrane (stage 10), cut nearly tangentially, to show the pores or annulae.

PLATE 6

FIG. 20. Internal yolky cytoplasm of fertilized egg in early blastema stage.

FIG. 21. Pole cell at syncytial blastema stage. The nucleus *N* is surrounded by a clearly double membrane (*N.M.*). The cytoplasm contains epsilon granules (ϵ) as well as mitochondria (*Mit.*); the surface no longer forms lobulations on fixation.

FIG. 22. Cytoplasm half-way between outer surface and centre of egg, early cleavage stage, to show fusion of beta granules or droplets (β) into larger masses, and one of the forms which may be assumed by the alpha granules (α) when imperfectly fixed.



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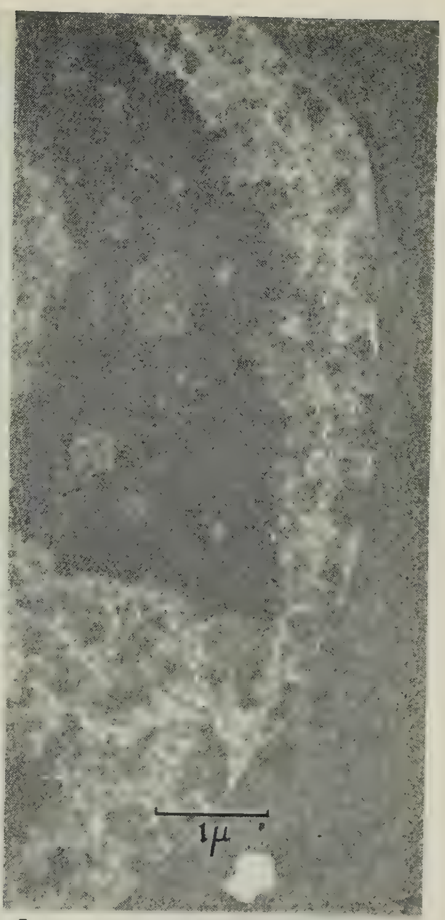

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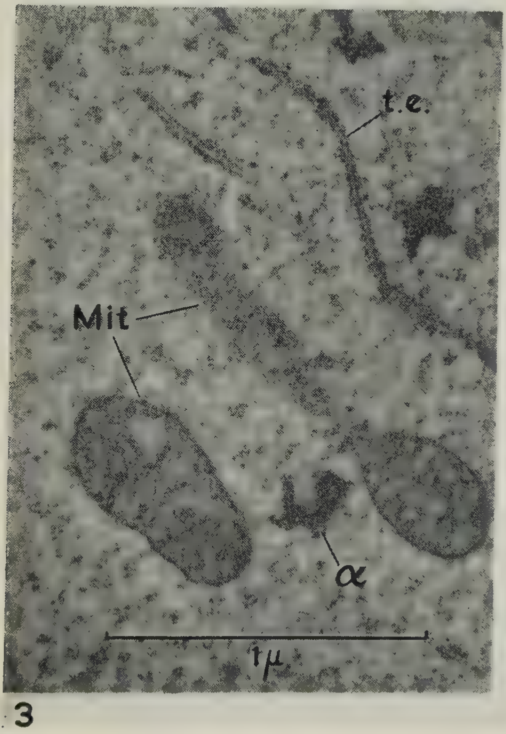

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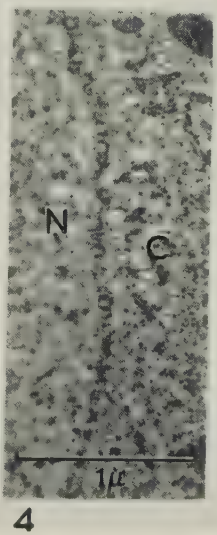

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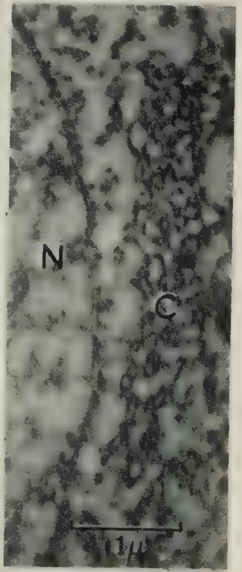

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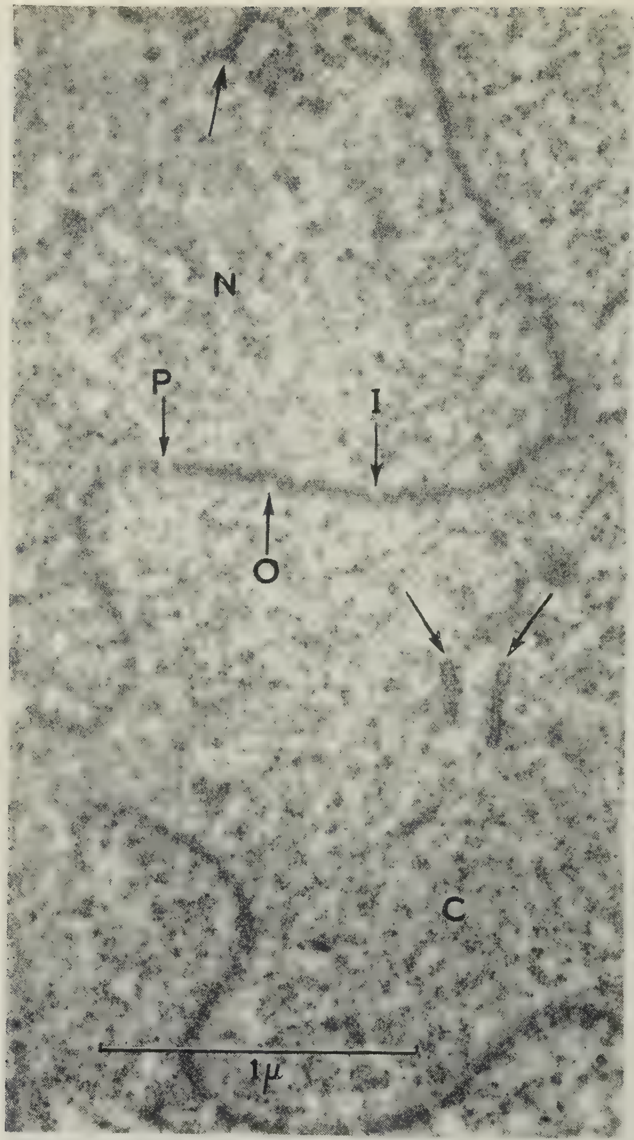
Plate 1



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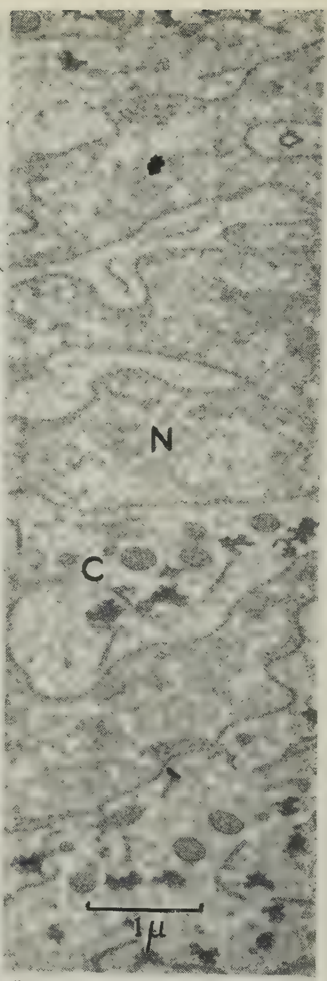

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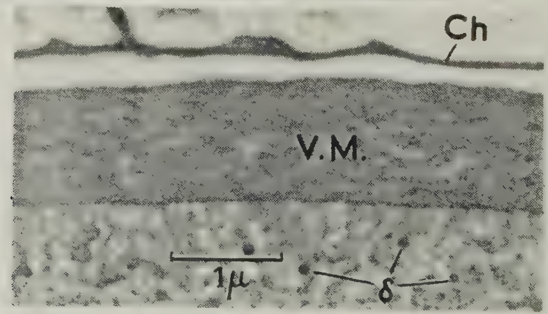

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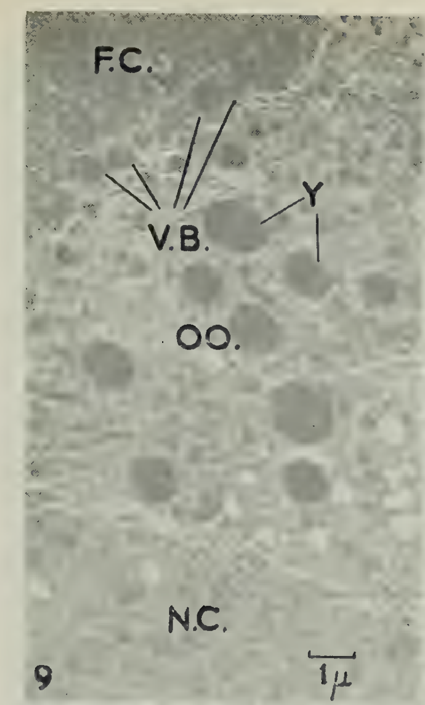
E. OKADA *and* C. H. WADDINGTON

Plate 2



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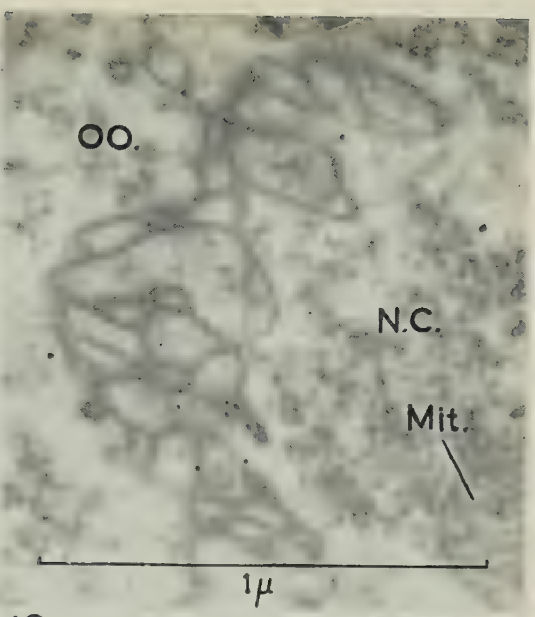

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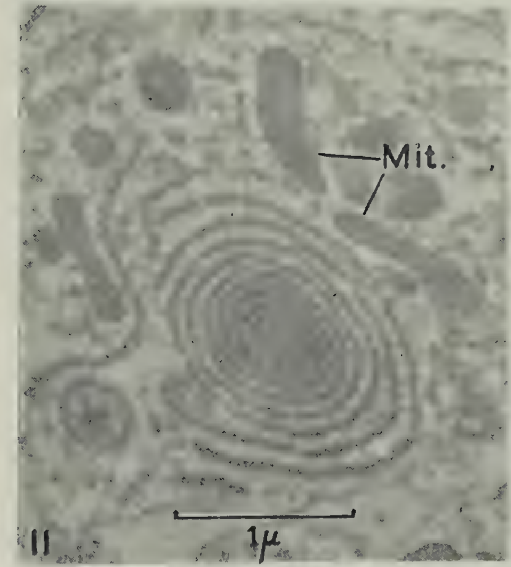

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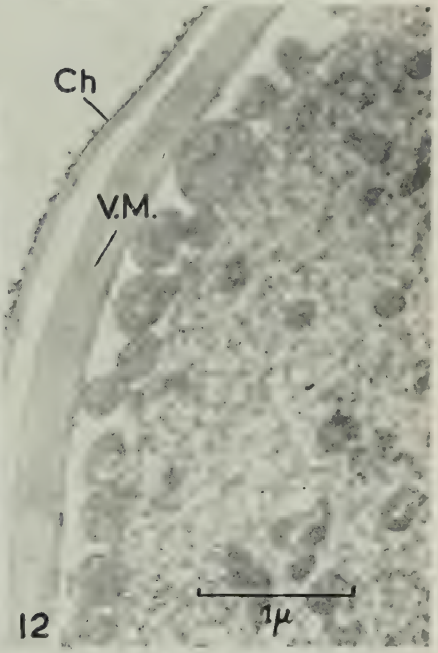

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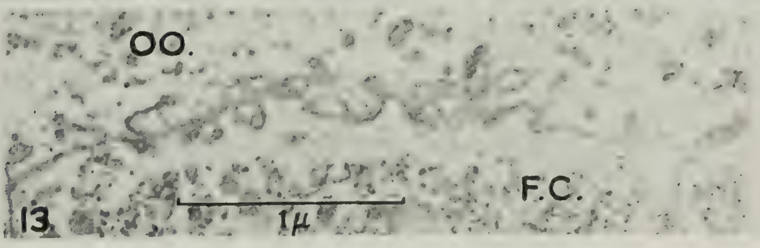

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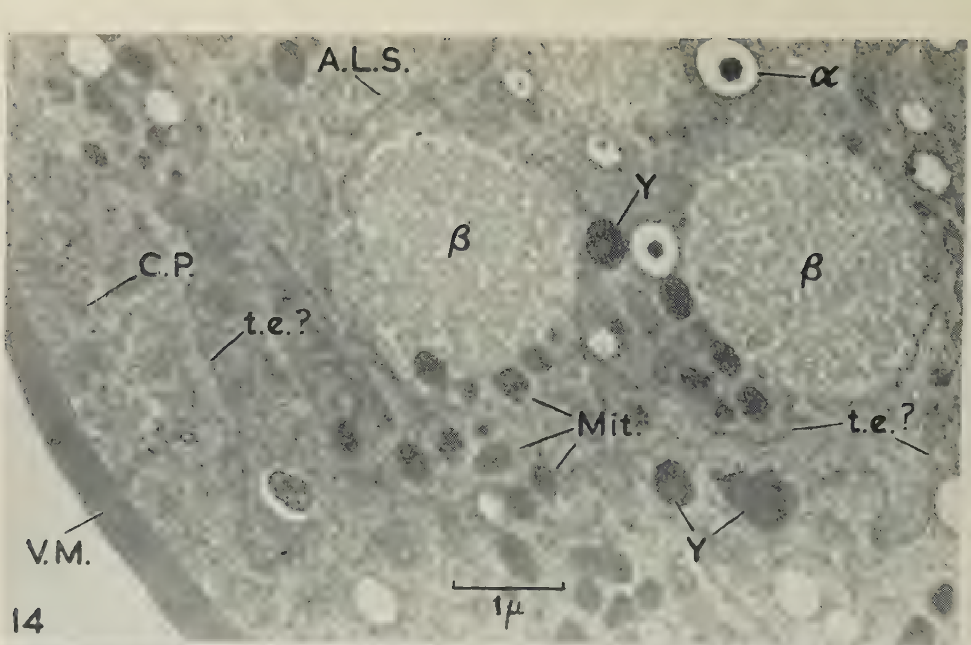

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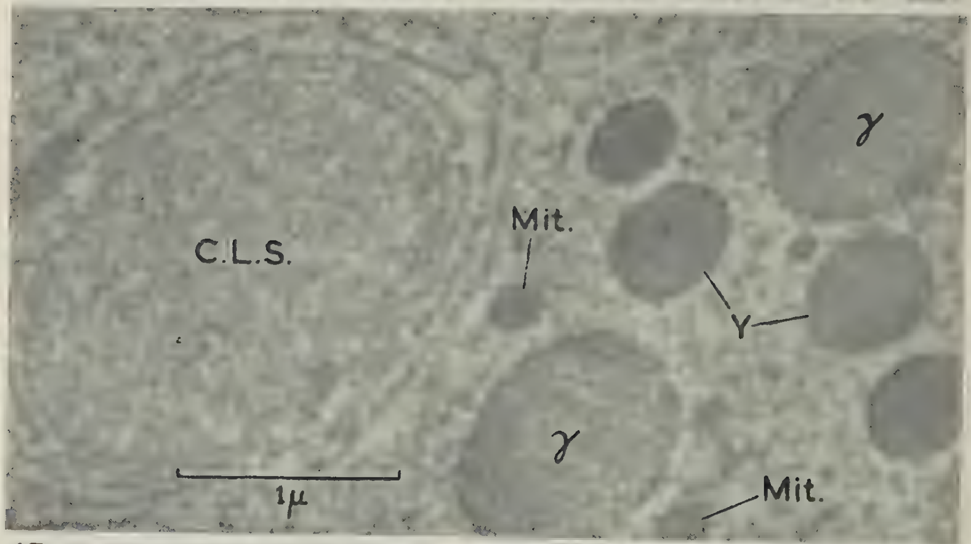

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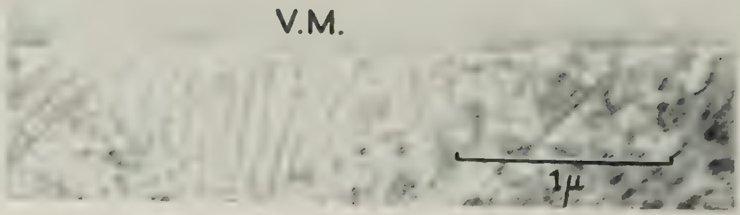

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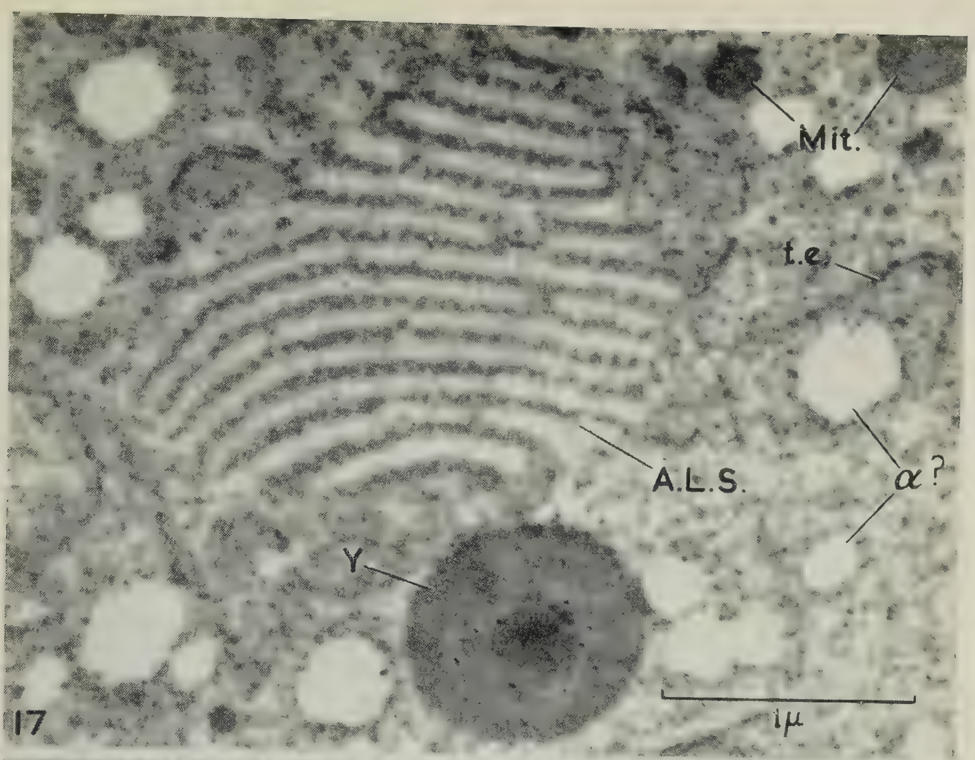
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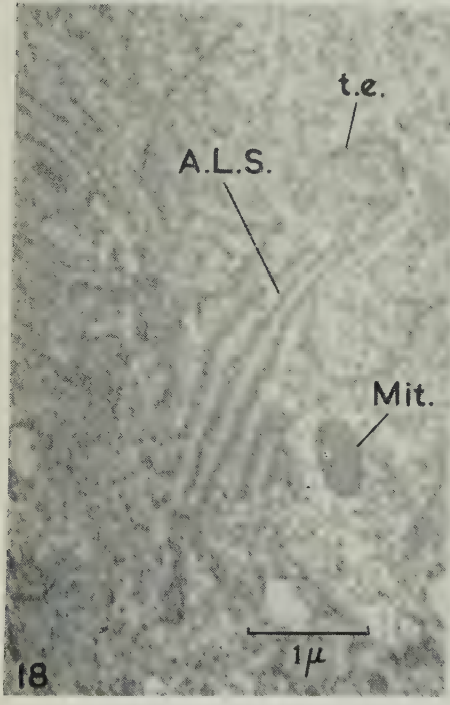
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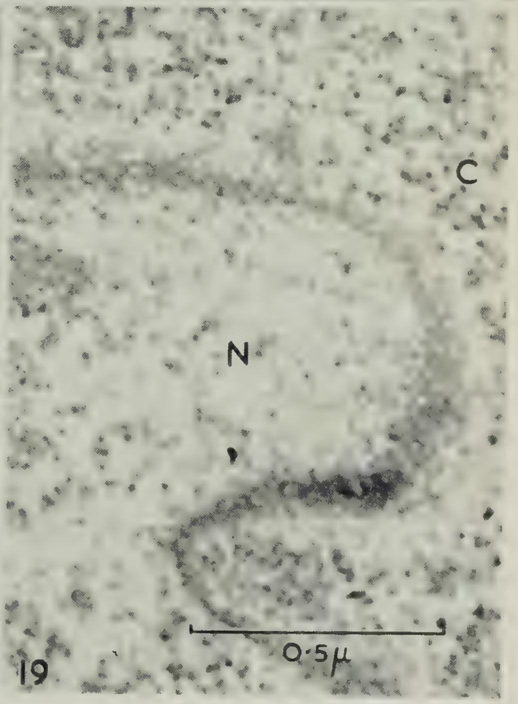
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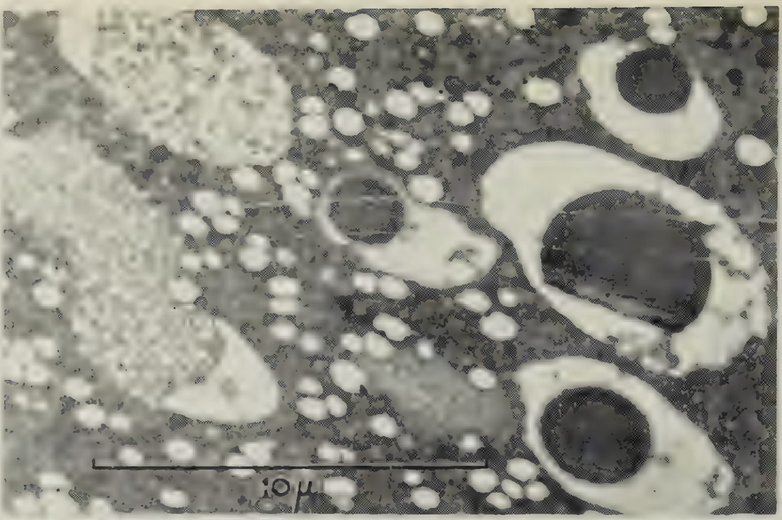

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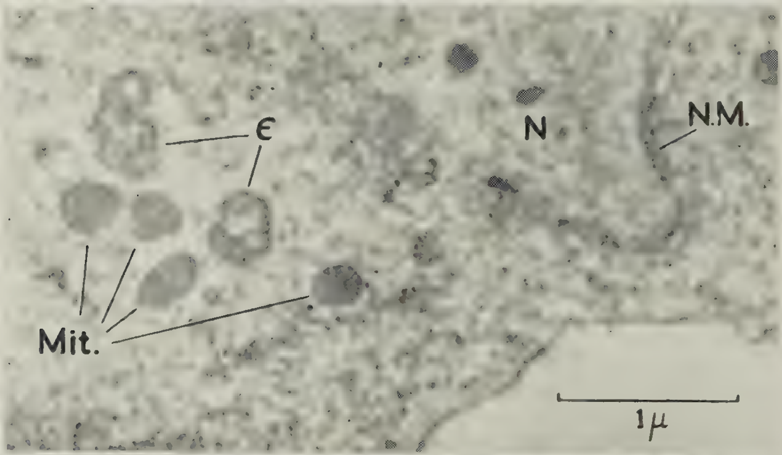

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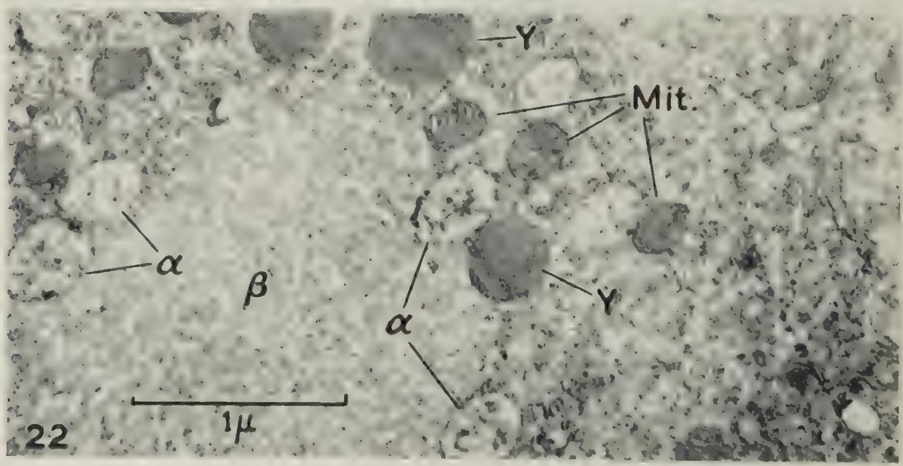

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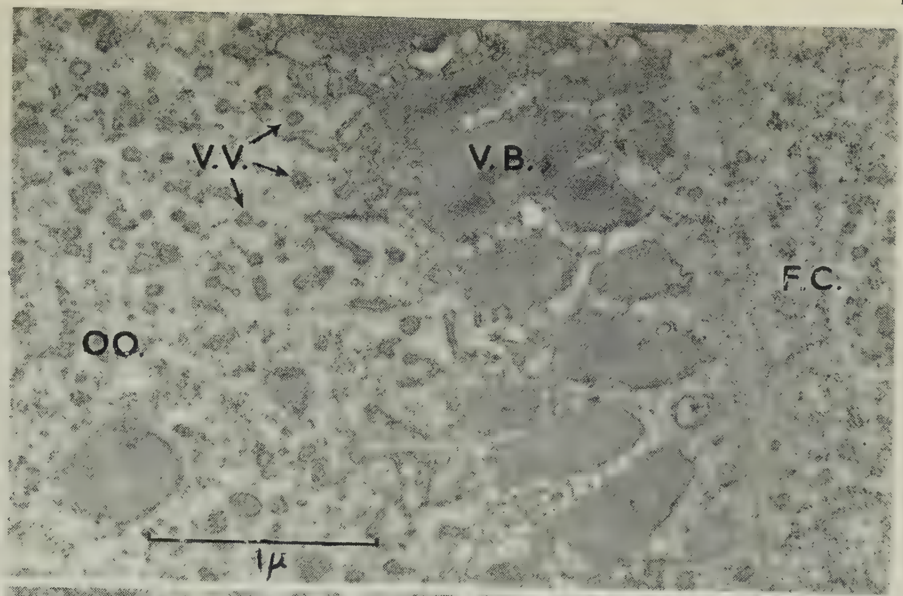

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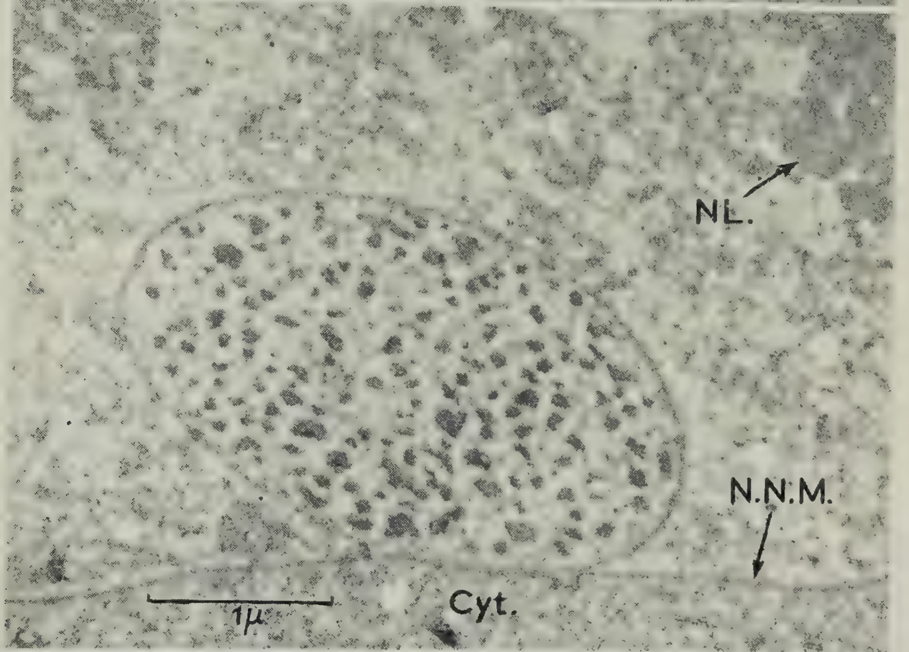

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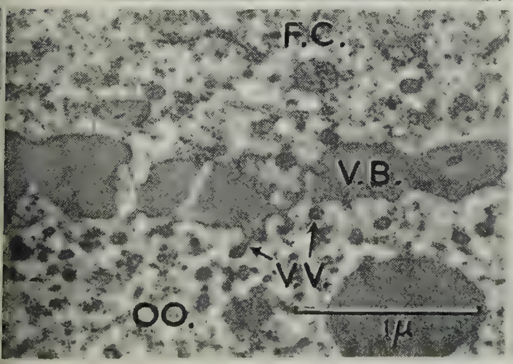

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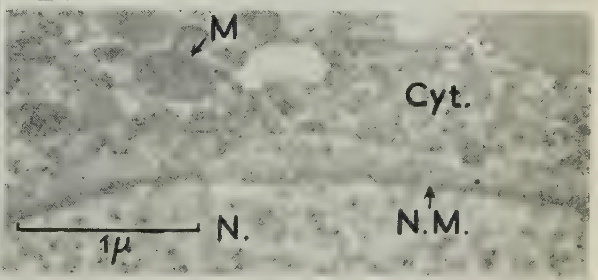

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Plate 7

PLATE 7

FIG. 23. Vitelline bodies (*V.B.*) forming between the oocyte (*OO.*) and a follicle cell (*F.C.*), stage 9. Note the vitelline vesicles (*V.V.*) in the ooplasm. The section is at an acute angle to the plane of the oocyte–follicle cell boundary.

FIG. 24. Part of nurse-cell nucleus, stage 9, showing an ovoid inclusion of undetermined nature. *N.N.M.* is the nurse-cell nuclear membrane, *NL* nucleolar material.

FIG. 25. Vitelline bodies (*V.B.*), stage 9; section approximately perpendicular to oocyte (*OO.*)–follicle cell (*F.C.*) boundary.

FIG. 26. Membrane (*N.M.*) between nucleus (*N*) and cytoplasm (*Cyt.*), stage 9 oocyte, to show the finely granular layer immediately outside the nucleus. *M*, mitochondrion.

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